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Comparison of Antibiotic Sensitivity Profiles, Molecular Typing Patterns, and Attribution of Salmonella Enterica Serotype Newport in the U.S., 2003-2006

Nehal Jitendralal Patel

COMPARSION OF ANTIBIOTIC SENSITIVITY PROFILES, MOLECULAR TYPING PATTERNS, AND ATTRIBUTION OF *SALMONELLA* ENTERICA SEROTYPE NEWPORT IN THE U.S., 2003-2006

by

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ABSTRACT

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Comparison of Antibiotic Sensitivity Profiles, Molecular Typing Patterns, and Attribution of *Salmonella* enterica Serotype Newport in the U.S., 2003-2006
(Under the direction of Dr. Karen Gieseker)

Salmonella causes gastrointestinal illness in humans. The purpose of the study was to determine the relative contribution of different food commodities to sporadic cases of salmonellosis (attribution analysis) caused by *Salmonella* Newport (*S*N) using Pulsed-Field Gel Electrophoresis (PFGE) patterns and antimicrobial sensitivity (AST) data submitted by public health laboratories and regulatory agencies from 2003 to 2006. The genetic relationship between isolates from non-human (348) and human (10,848) sources was studied by two unique clustering methods: UPGMA and Ward. Results show poultry was the highest contributor of human *S*N infections, followed by tomatoes and beef. Beef was the largest contributing food commodity of multi-drug resistant (MDR)-AmpC infection patterns. Results from this pilot study show that PFGE and AST can be useful tools in performing attribution analysis at the national level and that *S*N MDR-AmpC patterns are decreasing and seem to be restricted to isolates from animal sources.

INDEX WORDS: Attribution analysis, *Salmonella* Newport, PFGE, antimicrobial sensitivity testing

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LIST OF ACRONYMS & ABBREVIATIONS

APHL	Association of Public Health Laboratories
AST	Antimicrobial sensitivity testing
CDC	Centers for Disease Control and Prevention
CSTE	Council of State and Territorial Epidemiologists
FDA	Food and Drug Administration
FDA-CFSAN	Food and Drug Administration's Center for Food Safety and Applied Nutrition
FDA-CVM	Food and Drug Administration's Center of Veterinary Medicine
FDA-ORA	Food and Drug Administration's Office of Regulatory Affairs
MDR	Multi-drug resistant
MDR-AmpC	Multi-drug resistant phenotype characterized by resistance to nine different antimicrobials: ampicillin, amoxicillin-clavulanic acid, cefoxitin, cephalothin, ceftiofur, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline
MIC	Minimum inhibitory concentration
NARMS	National Antimicrobial Resistance Monitoring System
PFGE	Pulsed-field gel electrophoresis
TIFF	Tagged Image File Format
UPGMA	Unweighted Pair Group Method with Arithmetic
USDA	United States Department of Agriculture
USDA-AMS	United States Department of Agriculture's Agricultural Marketing Service
USDA-ARS	United States Department of Agriculture's Agricultural Research Service
USDA-ERS	United States Department of Agriculture's Economic Research Service
USDA-FSIS	United States Department of Agriculture's Food Safety Inspection Service

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CHAPTER I

INTRODUCTION

The number of multi-state and international outbreaks of foodborne illness has increased in the recent decades due to the globalization of food markets and changes in food processing and distribution practices. Food may be produced in one country and be consumed and cause disease in a different country. Today, foodborne infections do not respect borders (Ribot, 2006). One of the leading causes of foodborne infections in the world including the U.S. is the bacteria *Salmonella*, which causes a gastroenteritis infection known as salmonellosis. Every year an estimated 1.4 million cases of salmonellosis lead to 16,000 hospitalizations, nearly 400 deaths, and cause a major healthcare burden on the U.S. economy (Mead, 1999). *Salmonella* Newport is one of the major serotypes of *Salmonella* and the topic of this thesis. It causes more than 100,000 infections annually in the U.S. (Greene, 2007).

Foodborne illnesses may have many sources. Virtually any food may contain foodborne pathogens. *Salmonella* is a zoonotic pathogen, which means that it has its natural reservoir in animals--often the gastrointestinal tract--and can be transmitted to humans through direct contact or by consumption of meat or food contaminated with fecal matter from animals (Heymann, 2004). If the broad geographic distribution of food is also considered, it is not difficult to understand that detecting foodborne outbreaks and identifying their sources may be challenging. A major challenge of rapidly detecting an outbreak is overcome by continuously monitoring the occurrence of foodborne pathogens isolated from sick patients by using highly discriminatory methods that can differentiate

isolates from sources, i.e. a common source outbreak, from all other isolates circulating in the community. Currently, this is done by subtyping all or nearly all *Salmonella* isolated from people in the U.S. in the PulseNet network, which is coordinated by the Centers for Disease Control and Prevention (CDC) and the Association of Public Health Laboratories (APHL). The subtyping method used in this network is called pulsed-field gel electrophoresis (PFGE) (Gerner-Smidt, 2006). A more thorough description of PulseNet and PFGE will follow in Chapter II.

Study Rationale

Many foodborne outbreaks of *Salmonella* Newport have involved multiple states at the same time and have been caused by a variety of food products, the most important being fresh produce and ground beef (Greene, 2007). In order to control infectious diseases, antimicrobial agents have been widely used in human and animal populations. In agriculture, antimicrobials are currently being used for therapy, disease prevention, and growth promotion (Lopes, 2006). Whenever antimicrobials are used, bacteria that were previously susceptible can develop resistance towards them at some point in time. During the last decade, multi-drug resistant (MDR) *Salmonella* Newport has emerged in American dairy cow production (Zhao, 2003). This is a major public health problem because these resistant strains have spread from their animal reservoir to cause disease in humans; and hence decreasing the number of effective antimicrobials to treat human infections.

The use of a genetic subtyping method such as PFGE eventually paired with antimicrobial susceptibility profiles may be used to obtain an understanding of routes of

transmission of salmonellosis and the potential sources of the infections during times of wellness, not restricted to outbreaks. It is essential to recognize the sources of illnesses in order to be able to implement efficient measures to prevent future illness. The data used in this thesis is obtained from the PulseNet *Salmonella* Newport database combined with antimicrobial susceptibility data from other CDC surveillance systems, the National Antimicrobial Resistance Monitoring System (NARMS), to determine the relative contribution of different food sources to infections caused by this pathogen (microbiological attribution analysis). In other words, food attribution analysis identifies which foods are vehicles for illness.

There are two different approaches to attribution analysis: epidemiological and microbiological. Epidemiological information from case-control studies of sporadic foodborne infections may be used as aggregated data. In case-control studies, patients that have been diagnosed with a foodborne infection are matched, usually on sex, age, and place of living, with healthy controls in the community; cases and controls are then interviewed with the same questionnaire focusing on known and potential risk factors for disease and food consumption for the week prior to the debut of disease (cases) or the week prior to the interview (controls). By comparing the answers from cases and controls it is often possible to identify risk factors and risk foods for the disease; however, case-control studies have limitations due to recall bias and immunity. For instance, if a relatively common infection expresses durable immunity, then an important part of the population may be immune and not susceptible to infection, which can impede associating exposures with illnesses. Epidemiological approach also utilizes outbreak investigation data, but the results only relates to outbreaks that have been investigated.

Microbiological approach compares subtyping data from various sources; for instance, subtyping data from animals, food, and humans to understand the impact of contaminated foods on public health (Batz, 2005). This thesis is an attempt to add more precision to the microbiological attribution analysis of *Salmonella* Newport infections by focusing on subtyping by PFGE and antimicrobial sensitivity testing.

The PFGE patterns and the resistance types of isolates from humans and animal and food sources present in the PulseNet and NARMS databases will be compared and related to American food consumption data. To do this, it is assumed that isolates from human infections will display identical or highly similar PFGE patterns and susceptibility profiles as isolates obtained from the plants and animals sources of these infections. The data used in this study are representative of *Salmonella* Newport's prevalence and geographical distribution in the U.S., and the data presents the trends from 2003 to 2006. This study is the first attempt to use this kind of data for attribution analysis of salmonellosis in the U.S. PulseNet data collected from 2003 to 2006 will be used in conjunction with NARMS antimicrobial susceptibility data from 2003 to 2004.

Study Objectives

The objectives of the study are:

1. Identify isolates of *Salmonella* Newport submitted to the PulseNet *Salmonella* database between 2003 and 2006.
2. Identify source type (human and non-human) of the submitted bacterial strains, and exclude isolates that do not have a known source type.

3. Divide foods into different food categories adopted from the epidemiologic attribution performed by CDC epidemiologists in land, plant, or sea categories. Land category includes the following foods: meat-poultry (beef, pork, poultry, reptiles, and equine), dairy, and egg. Plant category includes: produce (fruit-nuts and vegetables), grain-beans, and oil-sugar. Sea category includes finfish and shellfish (mollusk and crustaceans).
4. Assign names to the PFGE patterns of all the isolates in the database; first to non-human isolates and second to human isolates.
5. Enter antimicrobial sensitivity information of *Salmonella* Newport isolates submitted to NARMS from 2003 to 2004 in the PulseNet database.
6. Generate a dendrogram, or "genetic tree," to compare the patterns of the human and the non-human isolates in the PulseNet database in order to group isolates with similar or identical PFGE profiles.
7. Estimate the number and/or proportion of human infections that are attributable to various sources using this information and information about the consumption of the identified food categories in the United States.
8. Discuss the limitations and weaknesses of the study.
9. Propose ways to improve future attribution analyses.

Research Questions

1. What are the relative contributions of different food commodities to human infections caused by *Salmonella* Newport in the U.S. during 2003-2006?

2. Is multi-drug resistance (MDR) restricted to isolates with particular PFGE patterns and specific food sources or is MDR a universal phenomenon?

Hypothesis

1. DNA fingerprint patterns of isolates collected from non-human sources correlates and clusters with isolates collected from humans and will be useful for attribution analysis.
2. MDR is restricted to isolates specific to animal or food sources.

CHAPTER II

REVIEW OF LITERATURE

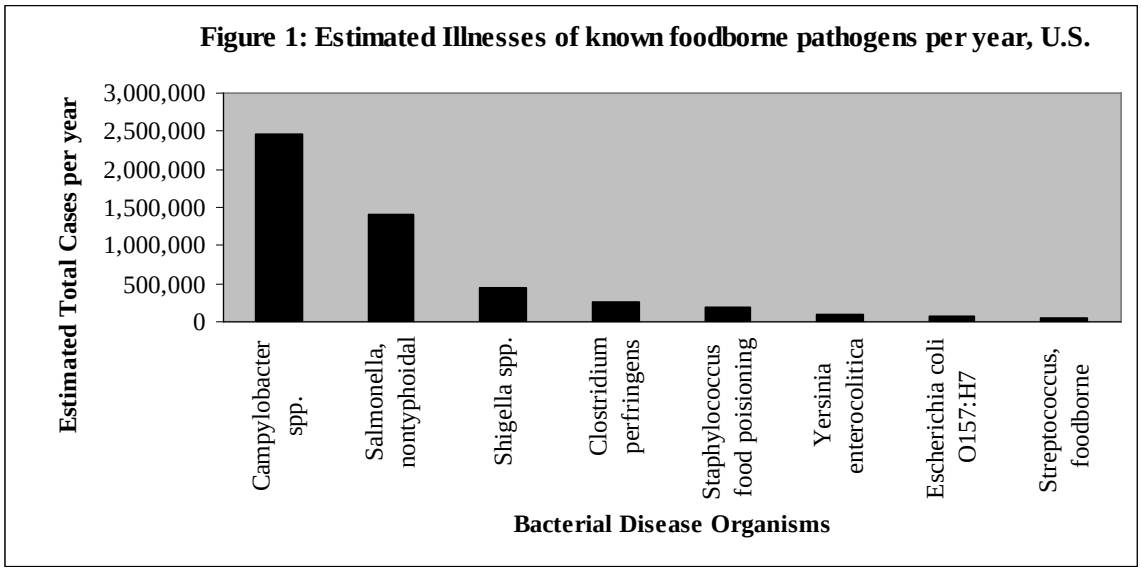
Overview

The review of literature for this study draws from the existing literature that focuses on the use of antibiotic susceptibility patterns and PFGE of *Salmonella* enterica serotype Newport in the U.S. The review covers the impact of foodborne illness and, more specifically, *Salmonella* bacteria on the U.S. The study reviews the financial impact of salmonellosis, and provides a background of *Salmonella* enterica, *Salmonella* Newport, and multi-drug resistant *Salmonella* Newport. Furthermore, the review also covers the Healthy People 2010 initiative; foodborne surveillance programs, including PulseNet, NARMS, and FoodNet; molecular subtyping techniques, including PFGE and antimicrobial sensitivity testing; attribution analysis; the Danish attribution model; U.S. outbreak data; previous studies; and the current study.

Food Illness: National Impact

Over 200 diseases are known to be transmitted through food. Illness can be caused by viruses, bacteria, parasites, toxins, metals, and prions. Symptoms of foodborne illness can range from mild gastroenteritis to life-threatening neurological, hepatic, and renal syndromes. It is estimated that in the U.S., foodborne microbial pathogens are responsible for approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year. Out of 76 million illnesses, known pathogens account for an estimated 14 million illnesses (Mead, 1999). Some of the major bacterial pathogens responsible for

illnesses are displayed in Figure 1. *Campylobacter* spp. causes the highest number of diagnosed bacterial foodborne infections in the U.S., and it can be transmitted to humans through water or food. The second highest cause of foodborne illness is *Salmonella* and *Shigella* spp. ranks a distant third (Mead, 1999).

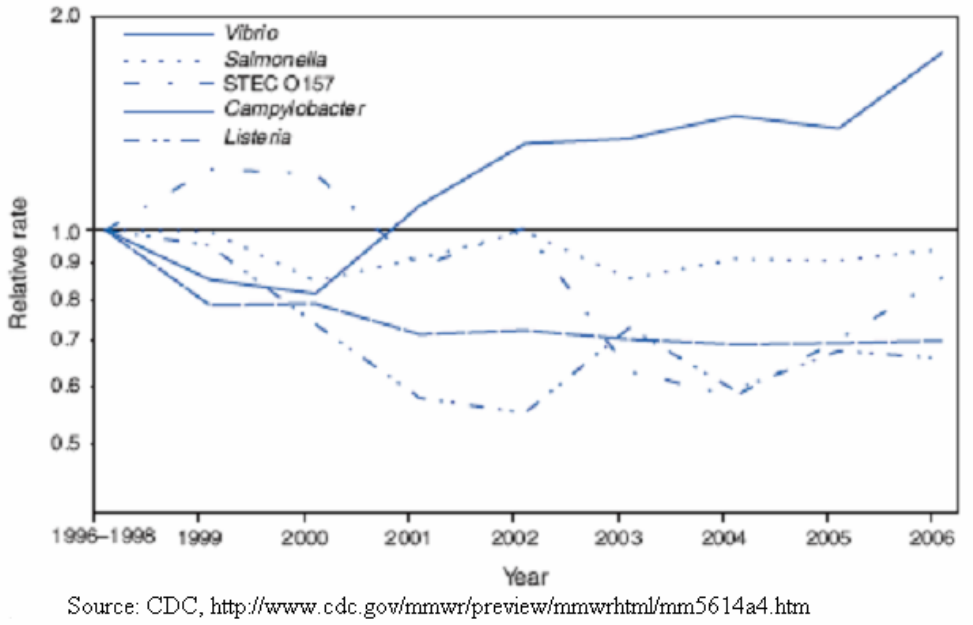


*Source: Mead, 1999

In the FoodNet report "Preliminary FoodNet Data on the Incidence of Infection with Pathogens Transmitted Commonly Through Food --- 10 States, 2006," preliminary population-based surveillance for laboratory-confirmed foodborne illnesses surveillance data for 2006 are compared with baseline data from the period 1996 to 1998 (CDC-MMWR, 2007). On one hand, incidence of infections caused by *Campylobacter*, *Listeria*, *Shigella*, and *Yersinia* has declined since the baseline period. On the other hand, incidence of infections caused by Shiga toxin-producing *Escherichia coli* O157 (STEC O157) and *Salmonella*, however, did not decrease significantly (Figure 2), indicating that

further measures are needed to prevent foodborne illness and achieve national health objectives (CDC-MMWR, 2007).

Figure 2: Relative rates compared with 1996-1998 baseline period of laboratory-diagnosed cases of infection with bacterial pathogens according to Foodborne Diseases Active Surveillance Network, 1996-2006



Source: CDC, http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5614a4.htm

Food Illness: *Salmonella* Impact

Overall Impact

The second most common bacterial cause of foodborne illness, and the focus of this thesis, is *Salmonella* enterica. Although salmonellosis is predominantly a foodborne disease, it can occasionally be acquired through contact to ill people or to pets, reptiles or contaminated drinking or recreational water. *Salmonella* is responsible for approximately 1.4 million illnesses per year in the U.S. (Mead, 1999). It is important to note that patients ascertained through laboratory-based public health surveillance represent only a fraction of all cases in the population. In fact, not all patients with diarrhea go to a clinician, and not all individuals seeking healthcare with diarrhea have stool cultures

done. Varma notes that physicians may be more likely to culture stool from a patient who has severe diarrhea, especially after international travel (Varma, 2006), and not all isolates cultured are submitted to a public health laboratory for further testing. It is estimated that on average only 1 out of 38 salmonellosis cases are reported (Mead, 1999). With these figures in mind, it is estimated that on a global scale, *Salmonella* is responsible for approximately 1.3 billion cases of acute gastroenteritis every year, resulting in 3 million deaths (Zhao, 2006).

Financial Impact

Foodborne illness is a significant public health problem in the U.S., and causes a heavy economic burden for the U.S. public and the healthcare system. United States Department of Agriculture's Economic Research Service (USDA-ERS) estimates the costs of illness and premature death for a number of foodborne illnesses, and these estimates have been used in regulatory cost-benefit and impact analyses. Like all cost estimates, the ERS estimates include assumptions about disease incidence, outcome severity, and the level of medical, productivity, and disutility costs. ERS estimates put the cost of *Salmonella* illnesses at approximately $2.4 billion total or about $1700 per case in 2006 in the U.S. (USDA-ERS, 2007).

Taxonomy

Salmonella is a genus of rod-shaped, gram-negative bacteria that has its natural reservoir in the intestine of animals. *Salmonella* bacteria are aerobic or facultatively anaerobic, and most are motile. *Salmonella* can persist for long periods outside their host, and may be found, for example, in sewage and surface water (Heymann, 2004).

More than 2,500 *Salmonella* serotypes that have been identified and reported. *Salmonella* serotypes are identified by their O antigens (somatic/cell wall) and H antigens (flagellar). The different antigens are numbered and divided into groups. The genus *Salmonella* is part of the family Enterobacteriaceae and is comprised of the species *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* has five subspecies, and *Salmonella* that infects humans and warm-blooded animals are *Salmonella enterica* subspecies enterica (Brenner, 1998).

Signs and Symptoms

Salmonella causes a bacterial disease called salmonellosis, which is usually manifested by an acute enterocolitis, with sudden onset of headache, abdominal pain, diarrhea, nausea, and sometimes vomiting. Fever is frequently present in salmonellosis patients. Dehydration may be severe, especially among infants and in the elderly. Even though infection may begin as acute enterocolitis, it may develop into septicemia or focal infection. Occasionally, bacteria may localize in any body tissue, producing abscesses and causing septic arthritis, cholecystitis, endocarditis, meningitis, pericarditis, pneumonia, pyoderma, or pyelonephritis. Deaths due to salmonellosis are uncommon, except in the very young, the very old, or the immunosuppressed (Heymann, 2004).

Transmission

Humans can become infected with salmonellosis by consuming contaminated water or food, especially animal products, such as eggs, meat, and milk, or vegetables that have been fertilized with contaminated manure or irrigated with contaminated water. Reptiles, such as pet turtles and iguanas, are particularly likely to harbor these bacteria, and direct contact with sources is a potential source of the infection. Fecal-oral

transmission from person to person can occur as well, especially when diarrhea is present. Furthermore, the bacteria can also be transmitted from human or animal carriers by unhygienic food preparation (Heymann, 2004). *Salmonella* is difficult to control in food animal environments because animals can be asymptomatic fecal shedders. Such "carrier" animals likely play an important role in the spread of infection between herds and flocks, and, therefore, serve as sources of food contamination and human infection (Zhao, 2007).

Along with food animals as a transmission source, *Salmonella* can also be transmitted to humans via produce. For instance, tomatoes have repeatedly been demonstrated as a vehicle in multistate *Salmonella* outbreaks (Hedberg, 1999; Cummings, 2001; CDC-MMWR, 2005; Greene, 2007). One DNA strain of *Salmonella* Newport has persistently caused illness from 2002-2006, and the same strain has been isolated from pond water used to irrigate tomato fields (Olson, 2007). These findings suggest that tomatoes were source of illness in all five years, and that there has been a stable, environmental reservoir in growing fields or production facilities (Greene, 2007; Olson, 2007). Furthermore, past *Salmonella* outbreaks due to contaminated tomatoes have been large and widely dispersed, which suggests that the contamination occurs early in the distribution chain, such as the farm or packing house, rather than at the consumer level (Greene, 2007). Guo and team have demonstrated that tomato stems and flowers inoculated with *Salmonella* can yield fruits contaminated with the bacteria when they have ripened (Guo, 2001).

Tomatoes are not the only produce that can transmit *Salmonella* to humans. Alfalfa sprouts have caused foodborne outbreaks in many countries around the world.

Ven Beneden, et al. reported an outbreak of *Salmonella* Newport that was associated with contaminated alfalfa sprouts. During the outbreak, bacteria with the outbreak DNA strain were isolated from almost all outbreak related cases and from leftover sprouts and seeds. (Van Beneden, 1999). Barak et al. showed that while *E. coli* was essentially rinsed from alfalfa sprouts with repeated washing steps, 1 to 2 log colony-forming units of *Salmonella* enterica remained attached per sprout. Particularly, *Salmonella* Newport strains remained adhered to 3-day-old sprouts (Barak, 2002). Research has shown that the reason alfalfa sprouts are a well-suited vehicle for salmonellosis is that alfalfa seeds are often stored for months or years under cool, dry conditions in which *Salmonella*e are stable (Bryan, 1968; Van Benden, 1999). Also, during the 3 to 5 day sprouting process, numbers of bacteria may increase 3 to 4 times and decrease little if at all during subsequent refrigeration (Andrews, 1982 and Jaquette, 1996, Van Benden, 1999). Since alfalfa sprouts are rarely washed or cooked before consumption, there is a greater risk of consuming the bacteria while eating the sprouts.

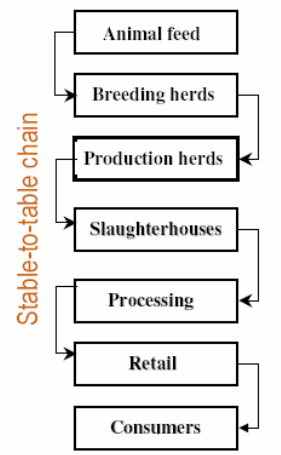
The continuous problem of *Salmonella* in produce highlights the importance of increasing awareness. The outbreaks explain that washing produce does not necessarily eliminate the bacteria; hence, it is necessary to understand reservoirs and routes of contamination and transmission to guide prevention strategies.

Prevention

According to the Centers for Disease Control and Prevention (CDC), approximately one in four Americans may experience some form of foodborne illness each year, and prevention of foodborne infections is fairly complex (Mead, 1999). Foods can become contaminated with pathogens at many points during the farm to table

pathway, and contamination can vary from pathogen to pathogen and over time (Figure 3).

Figure 3: Farm-to-table Chain Showing Possible Bacterial Interaction Source



*Source: Hald, T. "Human Illness Attribution: Concepts, Definitions, and Methods," at Workshop on source attribution of human zoonotic infections, Denmark, 2007

At the food animal industry level, there have been many approaches used to prevent and control salmonellosis, including improved biosecurity, vaccination, use of competitive exclusion products, and the introduction of novel immunopotentiators. However, these practices have had limited success so far. Due to this reason, the use of antimicrobial chemotherapy has been implemented in order to treat and control salmonellosis. This has led to increased antimicrobial resistance among several *Salmonella* enterica serovars (CDC-NARMS, 2006).

At the consumer level, Hillers and colleagues researched behaviors associated with prevention of foodborne illnesses. The use of a thermometer to cook foods adequately is most important for the prevention of illness caused by *Salmonella* species. The second most important behavior for the prevention of illness is to avoid cross-

contamination, followed by drinking only pasteurized milk and juices. Washing hands with soap and water before and after handling raw foods is also essential in preventing illnesses (Hillers, 2003). Even with information available that can enable consumers to make informed choices about food consumption and handling behaviors, the numbers of foodborne illnesses continues to be a significant health burden in the U.S.

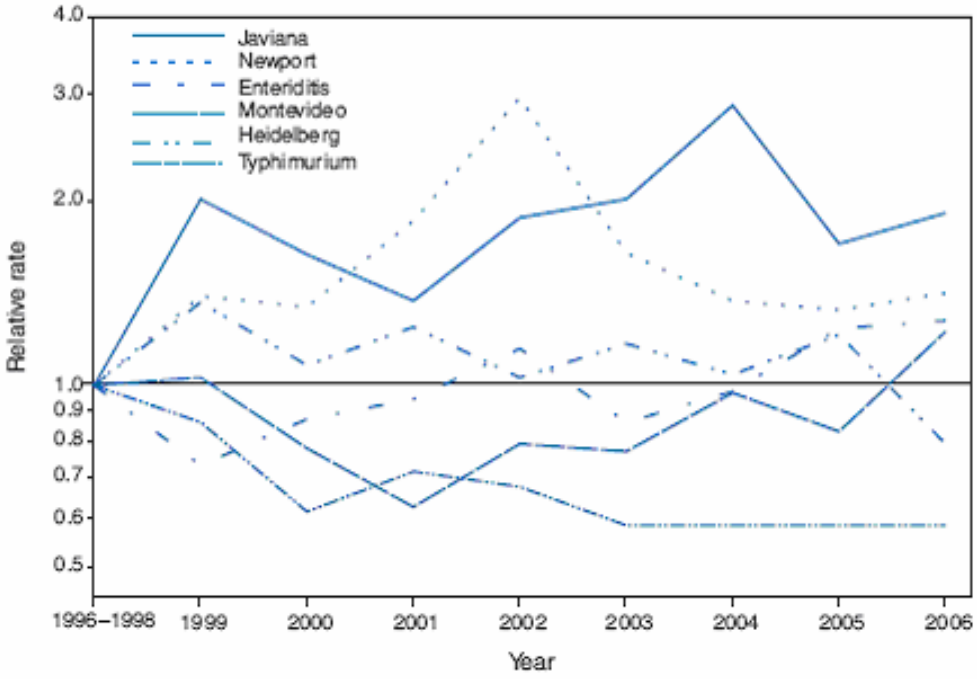
Treatment

Persons with diarrhea usually recover completely, although it may be several months before their bowel habits are entirely normal. Even though symptoms of salmonellosis are generally mild and last only a few days, salmonellosis can be extremely serious in the very young, the elderly, and/or immunocompromised individuals. Persons with severe diarrhea may require rehydration, often with intravenous fluids. Antibiotics are not usually necessary for treatment unless the infection spreads from the intestines, and in such cases the infection can be treated with ampicillin, gentamicin, trimethoprim/sulfamethoxazole, or ciprofloxacin. Unfortunately, some *Salmonella* bacteria have become resistant to antibiotics, largely as a result of the use of antibiotics to promote the growth of animals used for food . A small number of persons who are infected with *Salmonella*, will go on to develop pains in their joints, irritation of the eyes, and painful urination. It can last for months or years, and can lead to chronic arthritis which is difficult to treat. Antibiotic treatment does not make a difference in whether or not the person later develops arthritis (CDC-Salmonellosis, 2007).

Salmonella Newport (Multi-drug resistance and pan-susceptible)

Salmonella serotype Newport is the third most common cause of salmonellosis in the U.S. over the past 10 years, and causes more than an estimated 100,000 infections annually in the U.S. (Greene, 2007). According to FoodNet's surveillance data released in 2007, there is a significant increase in incidence compared with baseline levels for five out of six top *Salmonella* serotypes, including *Salmonella* Newport (Figure 4). Of the 5,957 (90%) *Salmonella* isolates serotyped, seven serotypes accounted for 64% of infections: Typhimurium (19%), Enteritidis (19%), Newport (9%), Javiana (5%), Montevideo (4%), Heidelberg (4%), and I 4,[5],12:i:- (4%) (CDC-MMWR, 2007). According to the data, *Salmonella* Javiana should be an increasing concern because number of illnesses caused by Javiana are rising. Very few sources or vehicles have been identified for *Salmonella* Javiana (Van Duyne, 2007, personal communication), and it is hard to utilize attribution analysis without having a confirmed source of infection. On the other hand, *Salmonella* Newport is known to be transmitted from various animal and produce sources (Rankin, 2002), and illnesses caused by *Salmonella* Newport can be utilized to perform attribution analysis. According to the CDC, there was a 12% increase in the incidence of human infections caused by *Salmonella* Newport from 1996 to 2003 (CDC-FoodNet, 2003).

Figure 4: Relative rates compared with 1996-1998 baseline period of laboratory-diagnosed cases of infection of the six most common Salmonella serotypes according to Foodborne Diseases Active Surveillance Network, 1996-2006



*Source: CDC, http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5614a4.htm

Parallel to FoodNet's report, another foodborne CDC surveillance program called PulseNet reported that outbreaks caused by *Salmonella* Newport have been gradually increasing for the past four years (Table 1).

Table 1: Number of *Salmonella* outbreaks Reported by PulseNet*

Year		Clusters Reported
2003		13
2004		19
2005		20
2006		32

*Source: CDC-PulseNet, 2007

Isolation of *Salmonella* Newport from various food products, including but not limited to potato salad, hamburger, chicken, precooked roast beef, ham or pork, fish and seafood, alfalfa sprouts (Rankin, 2002), tomatoes (Greene, 2007), and peanuts (Kirk, 2004) is a big public health concern. More specifically, the worldwide emergence of multi-drug resistant phenotypes among *Salmonella* Newport is of greater increasing concern. Multi-drug resistant *Salmonella* Newport has been spreading on an epidemic scale in both animals and humans throughout the U.S. (Berge, 2004; Zhao, 2003). Many of these *Salmonella* Newport strains exhibit a multi-drug resistant phenotype characterized by resistance to nine different antimicrobials: ampicillin, amoxicillin-clavulanic acid, cefoxitin, cephalothin, ceftiofur, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (commonly referred to as *Salmonella* Newport MDR-AmpC). Furthermore, these strains also demonstrate decreased susceptibility to ceftriaxone (Berge, 2004; Harbottle, 2006; CDC-NARMS, 2006), a critical antimicrobial used for treating invasive salmonellosis in children (Guerrant, 2001).

Previous research studies have suggested that dairy cattle are major reservoirs for MDR *Salmonella* Newport in the U.S. (CDC-MMWR, 2002; Rankin, 2002; Varma, 2006; You, 2006) and Canada (Poppe, 2006). Furthermore, multistate outbreaks of MDR *Salmonella* Newport during 1970s and 1980s were associated with the consumption of ground beef, especially from dairy cattle (Fontaine, 1978; Holmberg, 1984; Spika, 1987). A study done by You et al. showed that *Salmonella* Newport that has been shed from dairy cattle has a long-term survival rate (approximately 50-100 days based on the concentration load of bacteria) in manure or manure-amended soils, which indicate the potential risk for environmental spread and subsequent transmission. Many dairy

operation farms keep manure in storage for weeks or months prior to field application. Hence, it is possible that in farm settings where MDR *Salmonella* Newport infection is present, the organism could survive in manure storage and be applied to agricultural fields and increase the potential for dissemination beyond the farm boundaries. Field investigations of dairy farms infected by MDR *Salmonella* Newport have shown that the organism frequently leads to positive samples from locations that receive drainage from animal housing or manure storage areas, streams, and stream edges visited by cattle. Therefore, MDR *Salmonella* Newport does present a clear danger to the agricultural community, water resource, and the environment at large (You, 2006). If resistant foodborne bacteria are present in food animal species, then these bacteria may contaminate food products at the time of slaughter and be transmitted to humans through the food chain.

Antibiotic use preferentially eliminates nonresistant bacteria and increases the proportion of resistant bacteria that remains. Therefore, resistance of bacteria impacts the public health in such a way that it increases morbidity and mortality from treatment failures and increases healthcare costs as newer and more expensive antibiotics are needed to treat infections (Tollefson, 1998). Patients that have been infected with *Salmonella* Newport due to MDR-AmpC strains of bacteria tend to have more severe illness compared to patients with pan-susceptible strain bacterial infections. The severity could be due to the fact that infections occur disproportionately in patients that have an underlying immunosuppressive condition, such as HIV, steroid use, or an organ or bone marrow transplant (Devasia, 2005). Studies have shown that the strongest non-dietary risk factor for multi-drug resistant *Salmonella* Newport infection is taking antimicrobial

agents to which the bacteria is resistant during the 28 days prior to the onset of illness. According to Varma, et al., antimicrobial agents used during the 28 days prior to the onset of gastroenteritis illness among case patients with Newport MDR-AmpC infection included: amoxicillin for ear, sinus, throat, or upper respiratory tract infection; amoxicillin/clavulanate for ear, sinus, upper respiratory tract infection, and skin infection; cephalexin for skin infection; levofloxacin for bronchitis or pneumonia; penicillin for postsplenectomy; trimethoprim-sulfamethoxazole as prophylaxis in chemotherapy (Varma, 2006). It is important to rapidly identify drug resistance of bacterial strains to prevent and treat disease (Fontana, 2003).

While foodborne outbreaks caused by foods of animal origin tended to be MDR *Salmonella* Newport, outbreaks caused by contaminated produce tended to be pan-susceptible to antimicrobial agents (Greene, 2007). For instance, an outbreak caused by tomatoes grown and packed on the eastern shore of Virginia contaminated with a pan-susceptible *Salmonella* Newport strain sickened approximately 510 patients in 26 states in 2002. The same strain of *Salmonella* Newport caused illness in 2003, 2004, 2005, and 2006, and tomatoes were possibly the source in all five years. In 2005, an FDA traceback led to tomatoes grown on the eastern shore of Virginia, where the outbreak strain was isolated from pond water used to irrigate tomato fields in 2005. These strains of bacteria were pan-susceptible to antimicrobial agents (Greene, 2007).

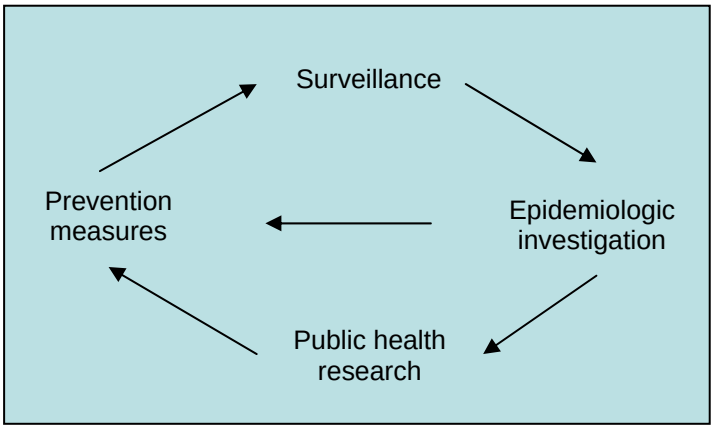
Foodborne Disease Surveillance

Healthy People 2010 Initiative

Even with information available that can enable consumers to make informed choices about food consumption and handling behaviors to prevent foodborne illness, the numbers of foodborne illnesses has caused a significant health burden in the U.S. For this reason, food safety is one of the priorities listed in the Healthy People 2010 initiative. The first two objectives of the food safety focus are to reduce infections caused by key foodborne pathogens and to reduce outbreaks of infections caused by key foodborne bacteria. The pathogens of target for these objectives are *Campylobacter*, *Escherichia coli* O157: H7, *Listeria monocytogenes*, *Salmonella* spp., *Cyclospora cayetanensis*, and *Toxoplasma gondii* (Healthy People 2010, 2000). In 2006, the overall incidence for *Salmonella* was 14.21 per 100,000 population (CDC-MMWR, 2007). The Healthy People 2010 objective for incidence of *Salmonella* infections for year 2010 is 6.80 per 100,000 population (Healthy People 2010, 2000).

Public health surveillance is critical to ensure health and safety of the people, to define the burden of infections, to track the trends in their incidence, and to detect outbreaks. Surveillance means monitoring specific infections diagnosed in a defined population. Surveillance followed by outbreak detection and investigation are important parts of a control strategy because they assist in determining the pathways that are most problematic as well as help to prevent new exposures and illnesses (Figure 5) (Tauxe, 2006).

Figure 5: The cycle of public health prevention



*Source: Tauxe, 2006

According to Healthy People 2010's Food Safety Initiative, the success of improvements in food production, processing, preparation, and storage practices can be measured through the reduction in outbreaks of disease caused by foodborne pathogens (Healthy People 2010, 2000). An outbreak is defined as a cluster of acute illnesses caused by a pathogen that are geographically and temporally associated, and occur in excess of what is usually expected for that time and place (Barrett, 2006). The increase of smaller outbreaks, which consist of fewer cases, may be a direct result of improved food preparation practices and better epidemiologic follow-up once cases are identified (Healthy People 2010, 2000).

The U.S. governmental agencies have developed programs that can help meet food safety objectives of Healthy People 2010. The United States Department of Agriculture (USDA) ensures the safety, wholesomeness, and accurate labeling of meat, poultry, and egg products (USDA-FSIS, 2004). The Food and Drug Administration (FDA) ensures the safety and wholesomeness of foods other than meat and poultry

(FDA-FS 01-2, 2005). The CDC monitors the rates of foodborne diseases in the U.S. and international countries, investigates outbreaks of foodborne illnesses, and facilitates efforts to prevent foodborne disease (Healthy People 2010, 2000). Program costs are paid by the U.S. government for now, and continuation of these funds is required to reduce infections caused by key foodborne pathogens and to reduce outbreaks of infections caused by key foodborne bacteria. The reduction of foodborne pathogens is necessary to meet the Food Safety Initiative objective of Healthy People 2010.

PulseNet

PulseNet is the molecular surveillance network for foodborne infections in the U.S. CDC's PulseNet program is a network of public health laboratories that subtype bacteria using standardized DNA fingerprinting methods and submit the results to an electronic database (Swaminathan, 2001). Since its inception in 1996, PulseNet has been instrumental in the detection, investigation, and control of outbreaks caused by shiga-toxin producing *Escherichia coli* O157:H7, *Salmonella* enterica, *Listeria monocytogenes*, *Shigella* spp., and *Campylobacter* bacterias. The PulseNet network has expanded to Canada, Europe, the Asia Pacific region, Latin America, and the Middle East. These independent networks allow public health officials to share molecular epidemiologic information in real-time, and enable rapid recognition and investigation of national and international foodborne disease outbreaks. PulseNet USA is a collaboration between the Association of Public Health Laboratories (APHL), the Centers for Disease Control and Prevention (CDC), the Council of State and Territorial Epidemiologists (CSTE), the Food and Drug Administration's Center for Food Safety and Applied Nutrition (FDA-CFSAN), Office of Regulatory Affairs (FDA-ORA) and Center of Veterinary Medicine

(FDA-CVM), and the U.S. Department of Agriculture's Food Safety Inspection Service (USDA-FSIS), Agricultural Research Service (USDA-ARS) and Agricultural Marketing Service (USDA-AMS). The participants in this network include public health laboratories in all 50 states, four counties, three cities, and eight food safety regulatory laboratories (Gerner-Smidt, 2006).

National Antimicrobial Resistance Monitoring System (NARMS)

The National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria was established in 1996, and is a collaboration between CDC, USDA-FSIS, USDA-ARS, and FDA-CVM. Participating health departments forward every twentieth non-Typhi *Salmonella* isolate, every *Salmonella* Typhi, as well as other organisms that are received at their public health laboratories to NARMS at CDC for sensitivity testing.

FoodNet

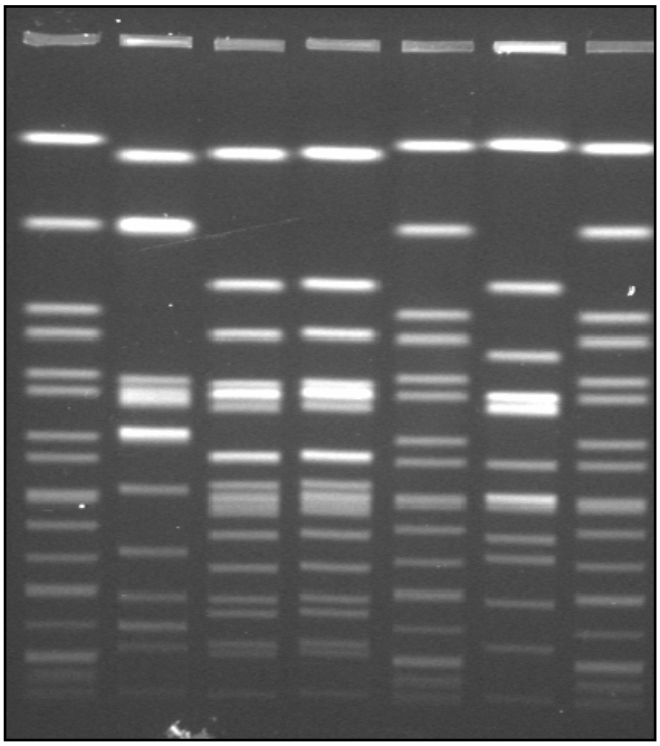
One of the principal foodborne disease components of CDC's Emerging Infections Program (EIP) is the Foodborne Diseases Active Surveillance Network (FoodNet). FoodNet is a collaborative project of the CDC, ten EIP sites, USDA, and FDA. FoodNet's duties consist of active surveillance for foodborne diseases and related epidemiologic studies designed to help public health officials better understand the epidemiology of foodborne diseases in the U.S. (CDC-FoodNet, 2007). The FoodNet surveillance program reported that *Salmonella* enterica serovars were the second leading cause of bacterial foodborne infections in 2004. Data from FoodNet and PulseNet show that *Salmonella* enterica Typhimurium, Enteritidis, and Newport are consistently the top three serotypes causing human infections in the United States (CDC-MMWR, 2003).

Techniques

Pulsed-field Gel Electrophoresis (PFGE)

Each type of bacteria has unique DNA which makes up a pattern of bands called a fingerprint. The fingerprints that laboratorians use to identify bacteria are called pulsed-field gel electrophoresis (PFGE) patterns. Laboratorians find bacterial fingerprints by cutting the bacteria's DNA into tiny pieces and then placing these pieces on a gel. The next step requires passing an electric current through the gel to separate the DNA pieces. Small pieces of DNA get carried farther down the gel than bigger pieces (CDC-PulseNet, 2007). This process creates a banding pattern or "fingerprint" that is shown in Figure 7.

Figure 6: Representative pulsed-field gel electrophoresis (PFGE) of seven *Salmonella* Newport isolates restricted with *Xba*I



*Source: PulseNet, CDC, 2007

PulseNet participating laboratories use standardized protocols developed and validated in CDC and public health laboratories to subtype bacteria. An ideal subtyping method would be 100% sensitive and specific, so all epidemiologically related isolates share the same DNA profile and all epidemiologically unrelated isolates would have a different DNA profile. In the laboratory and in the real world, there is no current method available that meets all of these criteria. However, PFGE does provide high levels of sensitivity, specificity, and reproducibility. PFGE is considered the "gold standard" for subtyping foodborne bacterial pathogens (Ribot, 2006). Molecular subtyping of isolates by PFGE has a great impact on public health. PFGE increases the ability of surveillance to identify outbreaks that otherwise might be overlooked, and hence increase the sensitivity (Tauxe, 2006). More specifically, subtyping can aid epidemiological investigations by identifying and tracking bacterial isolates, grouping illnesses by isolate, and positively identifying responsible food (Batz, 2005). PFGE also increases the specificity of the case definition, and therefore of the outbreak investigation at state and local levels and the findings (Tauxe, 2006). Certain pathogen subtypes can be associated with particular foods or animal sources, which enables illnesses from those subtypes to be similarly associated (Batz, 2005).

Antimicrobial Sensitivity Testing

Antimicrobial sensitivity testing involves the determination of the minimum inhibitory concentration (MIC) for antimicrobial agents. MIC is a quantitative method which identifies the minimum in-vitro concentration at which an antibiotic can inhibit growth. NARMS tests for the following 17 antimicrobial agents: amikacin, ampicillin,

amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. NARMS data can provide useful information about patterns of emerging resistance, which in turn can guide mitigation efforts. The data may also be an asset to outbreak investigations. Since antimicrobial use in food-producing animals may result in antimicrobial resistance that can be transmitted to humans through the food supply, antimicrobial resistance data from humans are important for the development of public health regulatory policy for the use of drugs in animals (CDC-NARMS, 2007).

DNA subtyping has been used to develop ideas about sources and to confirm a particular food as the culprit by subtyping pathogens from animals and from foods, which are collected as part of routine regulatory monitoring. Real-time subtyping of strains from foods and animals, and comparing the strains to human isolates help provide earlier warning of contamination in the food chain. Methods combining genetic DNA strain typing with antimicrobial susceptibility profiles are important epidemiological tools used to determine potential sources of infections.

Attribution Analysis

Food attribution is defined as the estimated incidence and valuation of illnesses caused by each pathogen, by percentage, to a set of food categories, to obtain estimated incidence and valuation of illnesses caused by each pathogen-food combination (Tick, 2003). In other words, food attribution analysis identifies which foods are vehicles for specific cases of illness. Attribution data is generally used to determine which foods

cause illness, what the illness trends are, and if regulation effects change. This information can be used to identify problems and patterns for public health officials and regulatory agencies to perform risk analysis, guide policy, and focus limited resources that are available. Hence, the people interested in such information would include consumers and food industry as well as public health and regulatory agencies (Ayers, 2007).

Researchers and regulators use various methods and data sources to attribute foodborne illnesses or risk of illnesses to specific pathogens in specific foods. Nonetheless, these approaches to food attribution are generally grouped into two broad categories: “microbiological” and “epidemiological.” Microbiological information includes data on microbes collected from humans and from animals and foods at various stages in the food production process. Microbial fingerprinting, such as PFGE, which uses markers to group similar pathogen subtypes, can be used to compare microbes from different sources and to link pathogen sources to contaminated foods or to specific cases of illness. This approach can provide focused information about single pathogens and about the range of reservoirs or foods that are included in comparative samples. Epidemiological information, either from data series of reported foodborne outbreaks or from case-control studies of sporadic cases, focuses on the final foods as consumed and may serve to link a broad variety of pathogens and foods or a single pathogen with a limited array of foods (Batz, 2005).

Denmark's Attribution Accounts

A leading country in foodborne attribution analysis is Denmark. In Denmark, healthcare cost burden lies on the government instead of the consumers; hence, there is no financial barrier preventing the citizens from seeking medical attention and reporting illnesses. Denmark has an integrated system with responsibilities incorporated in a network of agencies. All the data from public health surveillance and from pathogen monitoring on foods and animals are routinely collected, collated, analyzed, and reported by a single coordinating agency, the Danish Zoonosis Center. As a well-functioning entity, the center collects cultures from infected people, animals, and retail food sources. After the cultures are collected in Denmark, they are subtyped, which allows for direct comparison of surveillance and monitoring data and identification of public health outcomes by food source. There are three sources of foodborne illness surveillance data in Denmark: individual accounts and outbreak investigations of persons who report food poisoning to the public health officials; notifications by doctors and hospitals for all suspected infections; and reports by clinical microbiology laboratories of identified gastrointestinal pathogens. Denmark also performs regular food sources monitoring along the farm-to-table pathways—on farms, at slaughter houses, and on retail foods. Testing applies to all types of meats, dairy, and vegetable sources. All flocks of egg-laying chickens are regularly tested for *Salmonella* by a combination of serological and bacteriological methods. If a flock is positive for bacteria, then additional testing is performed for verification of infection. Every flock of broiler chickens, turkeys, and ducks is tested by a bacteriological test approximately three weeks prior to slaughter. Pig herds are continuously tested by serology, and herds that exceed a predetermined

proportion of seroreactors receive a follow-up bacteriological test. All of these animals are examined bacteriologically even after they are slaughtered. Dairy herds are examined serologically as well as categorized based on levels of antibodies. Lastly, fruits, vegetables, and shell eggs are surveyed at the retail level. After all, subtyping of isolated pathogens allow linkage between public health surveillance data and animal and food monitoring data (Batz, 2005).

Denmark uses several methods, including serotyping, phage typing, and PFGE methods to subtype isolates. With the available subtyping results, isolates from animals and humans are compared in a quantitative manner to assess the attribution of major animal reservoirs to human disease incidence. When human infections caused by *Salmonella* types are found in multiple reservoirs, then human infections are distributed proportionally to the occurrence of the distinctive types. One major flaw in this attribution method is that the method does not identify the causal infections implicated in individual cases of illness. Another flaw of the method is that it does not account for illnesses that are not unique to a particular animal which is not included in the list of monitored animals. The method also does not account for other sources that are capable of causing human illnesses, such as fish, pets, peanut butter, and water. The Danish model of food attribution assessment allows identification of reservoirs of infection in animal populations. However, the model does not identify various critical control points along the farm-to-table continuum, nor does it stimulate the effect of control strategies at these points. This model does not identify responsible foods at the point of consumption (Batz, 2005).

U.S. Outbreak Data

Unlike Denmark, the healthcare cost burden in the U.S. lies on the consumer and not with the government. High medical costs in the U.S. limit the actual number of reported illnesses; the number of reported illness is considerably less than the actual number of illnesses. There are several different foodborne illness surveillance systems in place at CDC, including PulseNet, FoodNet, and NARMS. With these surveillance systems, CDC conducts ongoing surveillance for the entire U.S., and foodborne outbreaks are investigated by public health labs in conjunction with the CDC. In the U.S., data is readily available for point-of-consumption food attribution, which allows outbreak data to be used to find sources of illness because outbreak data are observed at the public health endpoint and are therefore a direct measure of attribution (Batz, 2005). Outbreak data have implicated an array of food vehicles, i.e. *Salmonella* Tennessee in peanut butter (CDC-MMWR, 2007), *Salmonella* Newport in tomatoes (Greene, 2007), and *E. coli* in sprouts (Barak, 2002). Data can be used to systematically analyze trends, including antimicrobial susceptibility, temporal, and geographical prevalence trends. In Denmark, isolates taken from human, animal, and food sources are subtyped and compared to identify illnesses that are attributed by subtype to matching animal sources. On the other hand, in the U.S., subtyping is used to support outbreak investigation through data collected by PulseNet (Batz, 2005). Attribution using outbreak data indicates the relative importance of foods across all known etiologies (Ayers, 2007).

A pilot study done by PulseNet staff indicated that PFGE may be useful for microbiological attribution analysis of listeriosis. In the study, PulseNet participants performed PFGE on *Listeria* isolates from food, human, and environmental sources. The

five most common PFGE profiles from food isolates submitted for one year were determined, and then compared against human isolates in the database. The study showed that some PFGE profiles were almost exclusively associated with specific food commodities; for instance, the profiles that were largely associated with dairy products were not seen in any other food categories, with the exception of one pork isolate (Joyner, 2007).

Previous Studies

Previous studies have compared the prevalence and characteristics of *Salmonella* isolates from foods of non-human origins with the prevalence and characteristics of *Salmonella* isolated from humans. They have compared human surveillance data from the CDC as well as data from the USDA and FDA for *Salmonella* isolates in meat, poultry, eggs, produce, and seafood.

Fontana et al. have shown that clustering of PFGE patterns linked human and bovine cases, and PFGE detected associations helped epidemiologic investigations. Fontana's study only compared human and bovine isolates from Minnesota (Fontana, 2003); however, the study is useful in showing that PFGE provides a robust tool in characterizing the development of emerging pathogens.

Zhao et al. have shown that the antibiotic susceptibility profiles of *Salmonella* Newport correlate with PFGE clusters. The study showed that the presence of serotype Newport MDR-AmpC resistant strains in dairy cattle and finding indistinguishable Newport MDR-AmpC strains in animals and humans demonstrated that food animals can be a source of the pathogen, and emphasized the need to modify antibiotic dosing

practices and feed supplementation in animals. Zhao's team concluded that the overuse and misuse of antimicrobials may provide selective pressure for the spread of serotype Newport MDR-AmpC. The study was based on only 87 strains from 25 states from 2001 to 2002. Comparable to Zhao's study, this study will characterize *Salmonella* Newport isolates from humans and food animals using PFGE and determine their antimicrobial resistance phenotypes (Zhao, 2006). Unlike Zhao's study though, this study will compare surveillance isolates collected from 2003 to 2006, and also compare geographical trends and perform attribution analysis.

Tatavarthy et al. performed a study to determine if the correlation between PFGE and the antibiotic resistance profiles among *Salmonella* Newport isolates, as observed by Zhao and Fontana, could be found in another study group. However, Tatavarathy's group only used 30 *Salmonella* Newport isolates for study, and the isolates were collected from only two geographic locations: FL and WA. Hence, these isolates did not indicate the wide geographic region of the actual distribution of *Salmonella* Newport (Tatavarthy, 2006). Tatavarthy's study compared human and environmental isolates from two separate time periods. Therefore, it is difficult to form conclusions based on the study results that can be generalized to *Salmonella* Newport found in the U.S.

Varma et al. demonstrated that Newport MDR-AmpC infections in the U.S. were acquired domestically, most likely through the U.S. food supply of beef, egg, or chicken consumption, indicating bovine and poultry sources. The study concluded that *Salmonella* Newport MDR-AmpC infection is acquired through the U.S. food supply, and the source of infection is most likely from bovine and poultry, particularly among persons taking antimicrobial agents prior to infection. The study also indicated that international

travel was a risk factor for pan-susceptible *Salmonella* Newport infection (Varma, 2006). This study was based on a case-control study and not based on laboratory evidence. Case-control findings combined with laboratory results can be very instrumental in confirming Varma's findings.

Gupta et al. described a field investigation in New England that identified the emergence and epidemiology of new strains of *Salmonella* Newport MDR-AmpC by the organism. The investigation was based on a retrospective case-control study, and laboratory confirmation was received by analyzing PFGE and antimicrobial sensitivity data of the isolates. The results of the field investigation identified cattle on dairy farms as a reservoir for Newport MDR-AmpC. The infection with Newport MDR-AmpC in Massachusetts was domestically acquired and was associated with exposure to a dairy farm. Comparison of human and cattle isolates in a laboratory showed indistinguishable or closely related PFGE and antimicrobial sensitivity results. This emphasizes that the prevalence of ceftriaxone-resistant *Salmonella* infection has increased from 1998 to 2001 nationwide, and the primary reason for the increase was the emergence of Newport MDR-AmpC strains (Gupta, 2003).

The Current Study

In this study, the food attribution analysis will identify which foods are vehicles for specific cases of illness. Isolates subtyped from foods and animals are compared with the database of human isolates to determine the relative contributions of different food commodities to human infections caused by *Salmonella* Newport in the U.S. during 2003-2006. Furthermore, the study will compare antimicrobial sensitivity testing data of

Salmonella Newport to understand if MDR restricted to isolates with particular PFGE patterns or is MDR a universal phenomenon. This study will expand on Gupta's study and determine if the Newport MDR-AmpC is continuously causing salmonellosis in humans by analyzing data from 2003 to 2006, and also determine other sources of Newport MDR-AmpC that exist nationwide.

CHAPTER III

METHODS

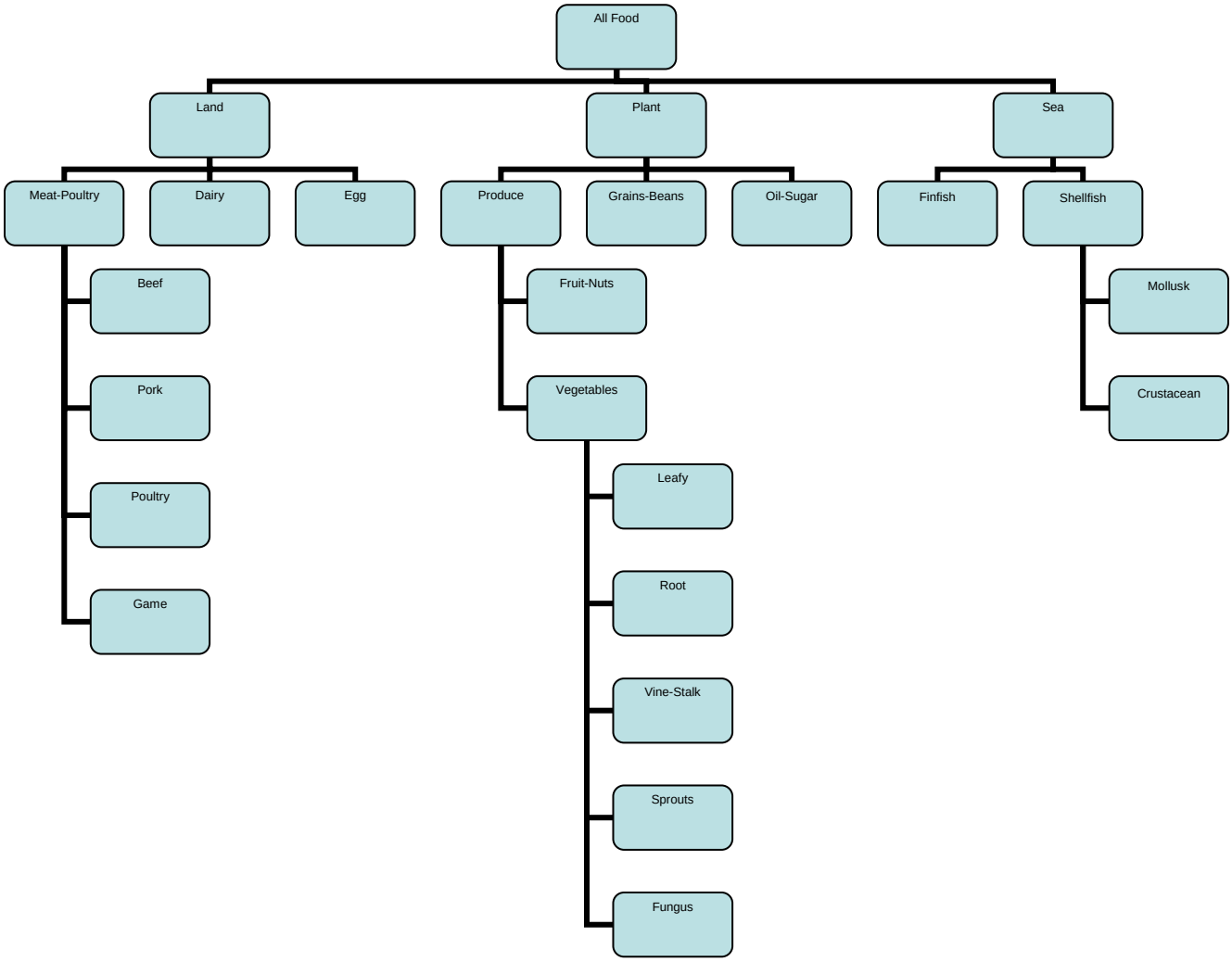
Institutional Review Board Application

The protocol title "Comparison of Antibiotic Susceptibility Profiles, Molecular Typing Patterns, and Attribution of *Salmonella* enterica Serotype Newport in the U.S., 2003-2006" was reviewed and approved by the Georgia State University Institutional Review Board on February 3, 2007. Protocol number is H07293.

Isolates of *Salmonella* enterica Newport

An isolate is a sample of bacteria. This study includes *Salmonella* Newport isolates obtained from various sources, including human, non-human sources such as animal, produce, and environmental isolates representing a variety of geographic regions within the U.S. Isolates were collected on random dates between 2003 and 2006. Foods implicated were categorized into major food commodities that are meaningful for regulatory agencies, industry, and consumers. Figure 6 displays the hierarchical scheme for classifying foods into food commodity categories. All food products were either divided into land, plant, or sea categories. The land category was further divided into meat-poultry, dairy, or egg. Meat-poultry were divided into the following categories: beef, pork, and poultry. The database also contains isolates from non-human animals, such as equine and reptiles. Isolates from an unknown source were not included in this study.

Figure 7: Hierarchical Scheme for Categorizing Food Items into Commodities*



*Source: Ayers, 2007

Microbiologic Methods

All isolates were serotyped as *Salmonella* Newport by the public health or federal laboratories submitting the isolates. Serotyping of *Salmonella* involves the

characterization of surface antigens, O and H antigens, according to the Kauffman-White scheme (Brenner, 1998). It is a common practice to initially test isolates with antisera to the most commonly encountered O groups. Once the isolate's O group is identified, most laboratories typically will test unknown isolates with antisera to H antigens found in commonly encountered serotypes within that particular O group. O antigens are characterized by a slide agglutination assay. Equal volumes (approximately 10 micro-liter) of a bacterial suspension and antiserum are emulsified on a glass slide. The slide is then gently rotated and observed against a dark background for evidence of agglutination. Visible agglutination is considered a positive agglutination. In the U.S., H antigens are characterized using a tube agglutination method. An overnight broth culture of the organism is first treated with formalin, next a sample of the formalin fixed broth culture is mixed with specific H antiserum and incubated at 50°C. The tube broth sample is then observed for flocculation. Tubes which remain clear following incubation are non-reactive with the tested sera. Tubes with visible flocculation are considered positive (Brenner, 1998). Usually, PulseNet participants streak isolates on blood agar plates. Then, well-isolated colonies are inoculated to triple sugar iron, lysine iron, and urea agar slants and incubated at 37 degree Celsius for 24 hours (Garrett, 2007).

PFGE Profiles

In the study, to determine the genetic relatedness of the isolates, patterns of isolates produced by PFGE were analyzed. Analysis was conducted for *Salmonella* Newport isolates collected from humans and non-human sources. Samples for PFGE that were prepared by PulseNet certified laboratorians were assumed to be prepared using a

CDC published procedure published by Ribot and team. This procedure recommends that the genomic DNA is prepared by embedding cells in agarose plugs and lysing the cells using lysozyme, sarcosyl, and deoxycholate. The DNA is digested in the agarose by using the restriction enzyme *Xba*I. The plugs are placed in a 1.2% agarose gel. The restricted fragments are separated by PFGE using 0.5 X Tris-borated-EDTA buffer at 14 degree Celsius and a Chef Dr III (Bio-Rad; Hercules, California, U.S.) gel apparatus. Conditions for electrophoresis are as follows: initial switch time, 2.2 seconds, final switch time, 63.8 seconds at an angle of 120 degrees at 6 Volts/centimeter for 20 hours. Restriction fragments are visualized by using an ethidium bromide stain, and the PFGE pattern is photographed, digitized, and saved as Tagged Image File Format (TIFF). These TIFFs are then analyzed using a customized software program called BioNumerics (Applied Maths, Sint-Martens Latem, Belgium). For PFGE, molecular-weight standards are run on each gel for normalization, which allows for comparison of PFGE from different labs (Ribot, 2006).

PFGE Pattern Naming

All PFGE profiles are assigned pattern names by the CDC PulseNet Team. A PulseNet standardized pattern name consists of 11 characters in the format: XXXYYY.####. The first three characters (XXX) represent the LITS code for the organism (i.e., JJP is the code for *Salmonella* Newport); the next three characters (YYY) represent the enzyme that was used to cut the DNA (i.e., X01 is the code that represents the enzyme *Xba*I); the four digits to the right of the decimal (####) are consecutive

numbers assigned to new profiles as they are detected. These numbers do not indicate any kind of relatedness between different PFGE types (Gerner-Smidt, 2006).

Antibiotic Sensitivity Testing

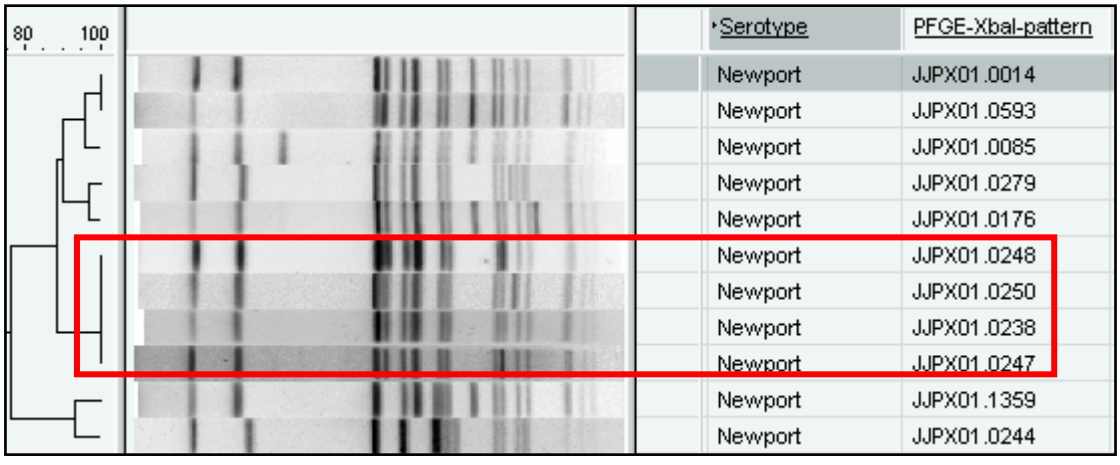
Antimicrobial minimum inhibitory concentration (MICs) for *Salmonella* serotype Newport isolates were determined by NARMS. Participating health departments forward every twentieth non-Typhi *Salmonella* isolate received at their public health laboratories to NARMS for susceptibility testing. The sensitivity testing involved the determination of the MICs for 17 antimicrobial agents: amikacin, ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole. The susceptibility of isolates was classified as being sensitive (S), intermediate (I), or resistance (R) according to Clinical and Laboratory Standards Institute (CLSI) standards (NCCLS, 2007). NARMS submitted their susceptibility testing results to PulseNet, and the results were analyzed via the customized software program BioNumerics.

Dendrogram Construction

A dendrogram, or bacteria family tree, places two isolates together in a genetic tree that are related based on band differences of PFGE fingerprints. TIFF images of DNA patterns and MICs of antibiotic resistance were analyzed by BioNumerics software version 4.01 (Applied-Maths, Sint-Martens-Latem, Belgium) using the Dice coefficient. The genetic relationship between isolates of non-human sources was studied by two

clustering methods: Ward with 1.5% position tolerance and UPGMA (Unweighted Pair Group Method with Arithmetic averages) with 1.5% position tolerance. These methods are pairwise clustering based on Dice algorithms that use a distance or similarity matrix as input (BioNumerics Manual, 2005). The position tolerance is the maximal shift (in percentage of the pattern length) between two bands that is allowed to consider the bands as matching. Position tolerance higher than 1.5% resulted in clustering of isolates that were visually not related. The optimal tolerance level to differentiate between two bands was 1.5% for this study. Use of two separate methods, Ward and UPGMA, ensures that isolates that are genetically related cluster together. For instance, in Figure 8, *Salmonella* Newport isolates with patterns JJPX01.0248, JJPX01.0250, JJPX01.0238, and JJPX01.0247 are more genetically related and hence they cluster together compared to isolates JJPX01.0014 and JJPX01.0593.

Figure 8: An illustration of a dendrogram created using Ward method and Dice coefficient calculations for *Salmonella* Newport isolates



Food Commodity Consumption

Food consumption data for each of the food commodities were acquired from USDA Economic Research Service (ERS) at http://www.ers.usda.gov/Data/FoodConsumption/FoodAvailQueriable.aspx. Food availability estimates measure food supplies moving from production through marketing channels for domestic consumption in the U.S. Per capita food availability data compiled by ERS reflects the amount of food available for human consumption in the U.S. These calculations are done annually by ERS, and provide estimates, for example, of the pounds of beef available for domestic consumption per capita per year. The data serve as surrogate for actual consumption. Use of this data is explained further in the "Attribution Analysis" section.

Attribution Analysis

The attribution analysis process was done by performing the following steps. First, unique PFGE patterns of non-human isolates were identified. The list generated consisted of one unique pattern for each food commodity. For example, if PFGE pattern JJPX01.0014 was submitted 36 times in four years from beef, but only one pattern of 36 isolates was included in the dendrogram. Furthermore, if pork and equine also isolated pattern JJPX01.0014, then one representative pattern of each commodity was included in the dendrogram or the genetic tree. Second, Ward cluster analysis was used to generate a dendrogram. Third, dendrogram was visually inspected and DNA fingerprint patterns were divided into clusters according to their relationship to other isolates. Fourth, clusters were confirmed using the second algorithm method, UPGMA. Among the two methods,

genetically related clusters were divided according to their branches. Patterns that were included in robust clusters by both methods were included as part of the study. In addition, patterns that only clustered by Ward method were not considered belonging to any clusters. Fifth, food commodity patterns were divided into major clusters and sub-clusters depending on their genetic relatedness. Sixth, unique representatives of food commodity isolates were compared to the unique pattern list of human isolates by including human unique patterns in the dendrogram of the food commodity isolates' cluster. The unique pattern list contains one example isolate of each pattern in the national database. Seventh, human patterns were divided into clusters and sub-clusters based on their genetic relatedness to food commodity patterns and the clusters or sub-clusters they belonged in. Patterns that clustered outside the defined clusters with non-human patterns were attributed to an unknown source. Next, numbers of human isolates belonging to each cluster or sub-cluster were calculated by counting number of isolates of each unique PFGE pattern of human isolate collected from 2003 to 2006, and assigning these isolates to their designated clusters or sub-clusters.

Once the isolates were assigned into groups, the next step of analysis required determining the actual amount of food commodities consumed per person in a year. Food consumption data for each of the food commodities were acquired from USDA-ERS. The amount of food consumed per person was divided by the number of isolates of each commodity to get pounds per capita per year per isolates, or, in other words, pounds per isolate of organism. This number was then used to get food per capita per cluster. Food per isolate of commodity was divided by the sum of food per capita for each cluster

and multiplied by the amount by number of human illnesses of each cluster to get numbers of illnesses attributed to food commodity for each commodity in each cluster

Attribution analysis with PFGE and antimicrobial sensitivity data required several steps. First, PFGE patterns that were classified as MDR-AmpC by NARMS were identified. After MDR patterns were identified, the next step was to determine the total number of human and food commodity isolates that were associated with PFGE patterns of MDR-AmpC isolates. Each commodity's MDR-AmpC isolates were divided from the total MDR-AmpC isolates to get attribution of MDR-AmpC patterns to the specific food commodity.

CHAPTER IV

RESULTS

PFGE: Non-human isolates

The non-human isolates (n=348) were submitted from the following sources during 2003-2006: beef (193), seafood (38), plant food (33), equine (28), pork (23), poultry (14), reptile (5), dairy (1), and other (13) (Figure 9). Items that were included in the "Other" category are avian, canine, feline, bat, and caprine. The isolates by category by year are shown in Table 2.

Figure 9: Percentage of Food Commodities in PulseNet database, 2003-2006

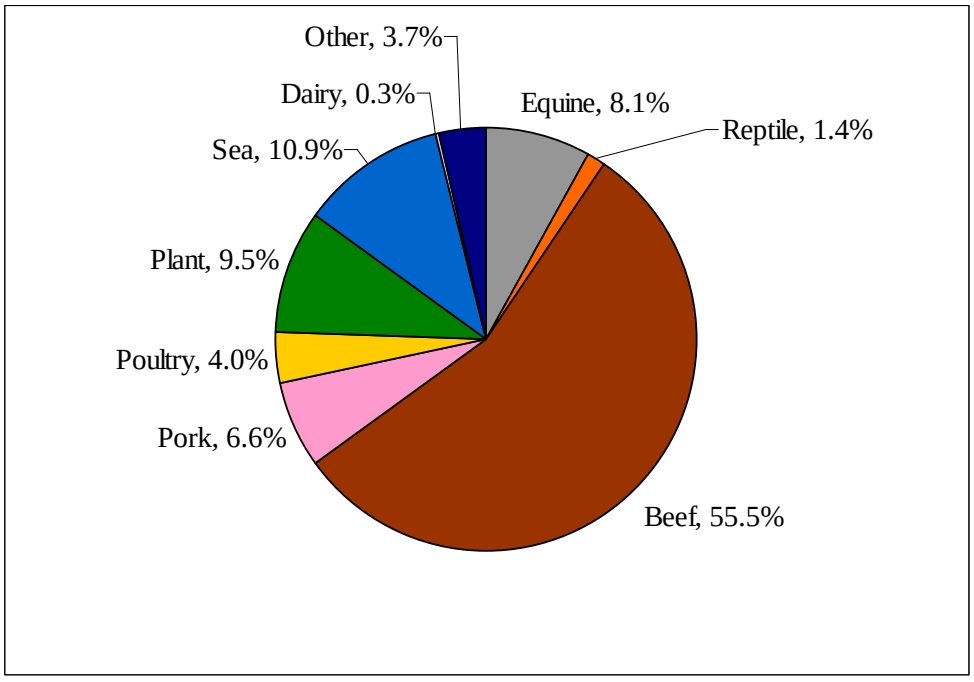
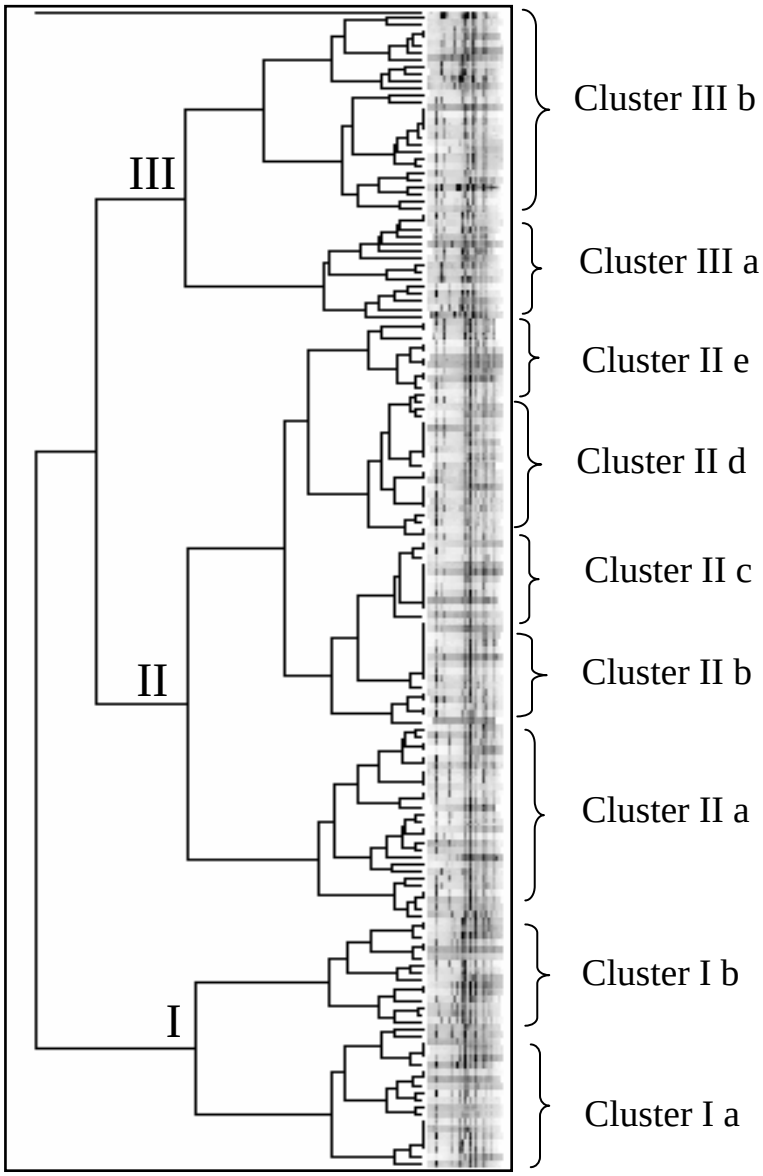


Table 2: Source and year of isolation of *Salmonella* Newport isolates					
Source	**Year isolated**				
	2003	**2004**	**2005**	**2006**	**Total no. isolated**
Beef	42	53	47	51	193
Dairy	0	0	1	0	1
Equine	3	17	8	0	28
Other	7	1	3	2	13
Plant	2	9	6	16	33
Pork	10	4	3	6	23
Poultry	0	5	8	1	14
Reptile	1	1	2	1	5
Sea	2	11	12	13	38
Total	**67**	**101**	**90**	**90**	**348**

PFGE analysis of the 348 isolates led to 162 unique patterns (defined as a unique pattern by food commodity); i.e. if pork and beef isolates both had PFGE pattern JJPX01.0014, they were both used to create clusters whereas if two beef isolates had PFGE pattern JJPX01.0014, then only one would be used for analysis. The number of isolates and number of unique patterns for each food commodity are displayed in Table 3. The 162 unique non-human PFGE patterns were then analyzed by Ward and UPGMA methodology to create a dendrogram. Three major clusters were identified: I, II, and III, which were then further classified into sub-cluster categories: Ia, Ib, IIa, IIb, IIc, IId, IIe, IIIa, and IIIb (Figure 10). There were 15 isolates with 11 different PFGE patterns that had long branches and did not fit into any one of these sub-clusters leaving 333 isolates for further calculations.

Table 3: Number of isolates and number of unique patterns of food commodities										
	Beef	**Dairy**	**Equine**	**Other**	**Plant**	**Pork**	**Poultry**	**Reptile**	**Sea**	**Total**
Isoaltes (n)	193	1	28	13	33	23	14	5	38	348*
Unique patterns (n)	56	1	14	10	21	16	7	5	30	162
*15 out of 348 isolates were excluded from calculations because they did not fit into any three of the clusters										

Figure 10: Ward dendrogram (Dice coefficient) calculated for 162 *Salmonella* Newport isolates collected from non-human sources



PFGE: Human isolates

From January 2003 to December 2006, 94,334 *Salmonella* isolates from humans were submitted to the National PulseNet *Salmonella* Database; 10,847 (11.5%) were serotype Newport. Distribution of the *Salmonella* Newport isolates compared to all *Salmonella* isolates is shown in Figure 11.

Figure 11: *Salmonella* Newport Relative to all *Salmonella* Isolates, 2003-2006

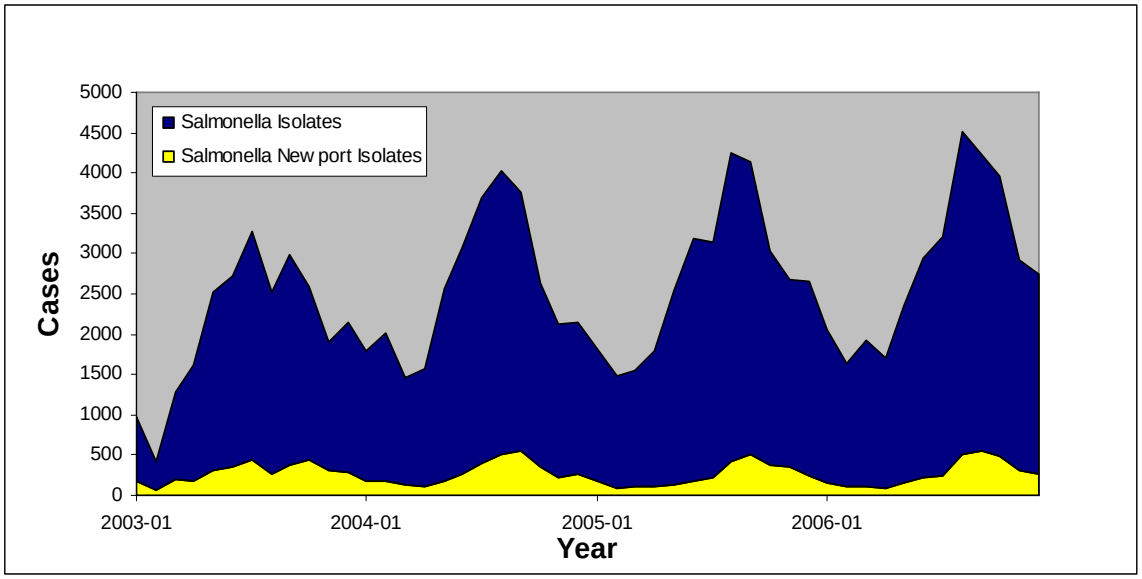
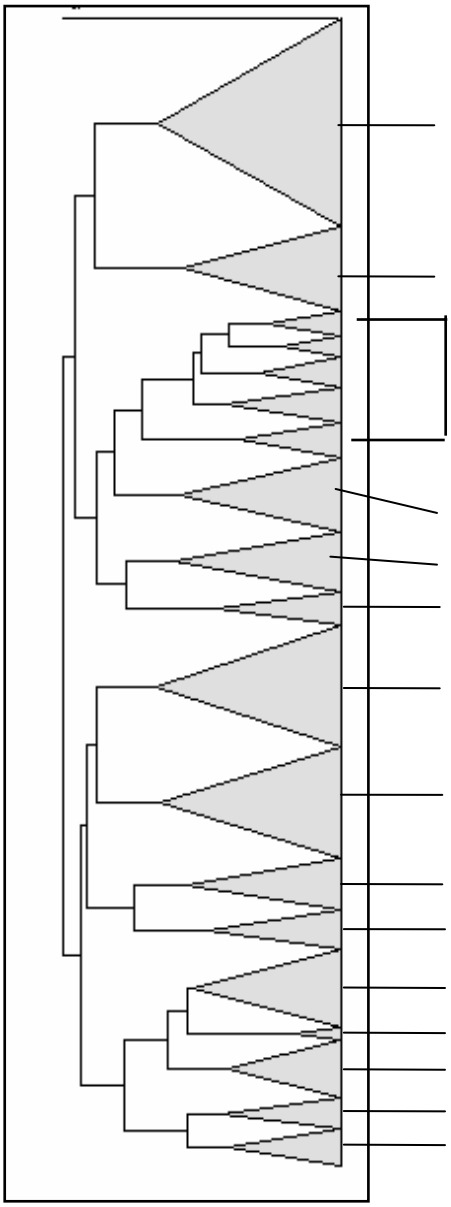


Table 4 shows characteristics of total *Salmonella* Newport isolates collected from humans by age, year, gender, and geographic regions.

Table 4: Total *Salmonella* Newport isolates by Age, Year, Gender, and Geographic Regions (n=10,847)	
Variable	**n=10,847**
Age, mean (range), years	33 (0-99)
Year	
2003	2,396 (22.1)
2004	2,899 (26.7)
2005	2,548 (23.5)
2006	3,004 (27.7)
Gender	
Male	4,065 (37.5)
Female	4,714 (43.5)
Unknown	2,068 (19.0)
Geographic Regions	
Northeast Central	1,106 (10.2)
Southeast Central	669 (6.2)
MidAtlantic	1,295 (11.9)
Mountain	675 (6.2)
New England	586 (5.4)
Pacific	871 (8.0)
South Atlantic	2,699 (24.9)
Northwest Central	919 (8.5)
Southwest Central	2,027 (18.7)

The next step of analysis required comparison of 162 unique representatives of non-human isolates to the unique patterns of human isolates. Out of 10,847 human isolates, there were 1,998 unique patterns. Comparison of 162 unique representatives of food commodity isolates to the 1,998 unique patterns from the human isolates by including human unique patterns in the dendrogram of the food commodity isolates' cluster led to a dendrogram that is illustrated in Figure 12.

Figure 12: A dendrogram representation showing genetic relationship of 1,998 human and non-human patterns



Human Cluster	Non-Human Cluster
A	IIIb
B	IIIb
C1	IId
C2	IIb
C3	IId
C4	IIe
C5	IIa
D	IIIb
E	IIIa
F	IIc
G	Ia
H	Ia
I	Ia
J	IIIb (little association)
K	Ib
L	Ib (little association)
M	Ib
N	Ib
O	IId (little association)

In the dendrogram, human patterns were compared to non-human patterns by overlaying human patterns on top of the non-human patterns, and they were categorized into sub-clusters according to their genetic relationship. The number of human isolates in

each cluster and sub-cluster was determined by figuring out the food commodity cluster for human clusters A through O. The total number of human isolates belonging to each cluster or sub-cluster was calculated by counting the number of isolates of each unique PFGE pattern of human isolate collected from 2003 to 2006, and assigning these isolates to their designated clusters or sub-clusters as displayed in Table 5. This table also shows the distribution of 333 non-human isolates in the appropriate food commodity category. There were 10,847 human *Salmonella* Newport isolates collected from 2003 to 2006, but only 9,445 isolates are listed in Table 5 because isolates for which PFGE patterns could not be assigned due to laboratory error while running PFGE were not included in further calculations.

Table 5: Number of non-human Isolates divided based on Food Commodities submitted to PulseNet and number of human isolates to the matching PFGE patterns, n (%)

Clusters	Beef	Pork	Poultry	Plant	Sea	Dairy	Equine	Reptile	Other	Human
Cluster I	**6 (3.3)**	**1 (4.5)**	**3 (21.4)**	**18 (58.1)**	**10 (27.0)**	**0 (0.0)**	**10 (37.0)**	**1 (20.0)**	**2 (20.0)**	**5642 (59.7)**
Cluster Ia	1	0	3	5	9	0	5	0	1	4,093
Cluster Ib	5	1	0	13	1	0	5	1	1	1,549
Cluster II	**177 (95.2)**	**20 (90.9)**	**6 (42.9)**	**3 (9.7)**	**4 (10.8)**	**0 (0.0)**	**17 (63.0)**	**0 (0.0)**	**6 (60.0)**	**1895 (20.1)**
Cluster IIa	72	7	0	0	0	0	13	0	0	454
Cluster IIb	14	1	4	0	4	0	1	0	2	390
Cluster IIc	5	1	0	0	0	0	0	0	1	55
Cluster IId	59	7	1	3	0	0	1	0	1	802
Cluster IIe	27	4	1	0	0	0	2	0	2	194
Cluster III	**3 (1.6)**	**1 (4.5)**	**5 (35.7)**	**10 (32.2)**	**23 (62.2)**	**1 (100.0)**	**0 (0.0)**	**4 (80.0)**	**2 (20.0)**	**1908 (20.2)**
Cluster IIIa	0	0	0	7	11	0	0	0	1	319
Cluster IIIb	3	1	5	3	12	1	0	4	2	1,589
Total Isolates	**186 (100)**	**22 (100)**	**14 (100)**	**31 (100)**	**37 (100)**	**1 (100)**	**27 (100)**	**5 (100)**	**10 (100)**	**9445 (100)**

The next step in the analysis required determining food consumption data. U.S. per capita food availability (pounds per capita per year) data compiled by USDA-ERS

reflect the amount of food available for human consumption in the U.S. In order to determine pounds per capita per year per isolate, the number of pounds per capita per year was divided by the number of isolates for each separate food commodity. The resulting pounds per capita per year per isolate are shown in Table 6. For plant sources, consumption of the two predominant sources present in the database, tomatoes and cantaloupes were considered. Therefore, only 16 out of 31 plant source isolates are used for attribution analysis because the other 15 plant derived isolates included pumpkin seeds, sesame seeds, kasoori methi, coriander, thyme, red chili powder, black pepper, soybean meal, and horchata, and no consumption data for these sources were available. The equine and reptile consumption in the U.S. is assumed to be negligible, and since no exposure or consumption information was available for these and other sources, these categories were not taken into account in further calculations.

Table 6: Number of human and non-human Isolates divided based on Food Commodities*

	Beef	Pork	Poultry	Plant**		Sea	Dairy***
				Tomatoes	Canteloupes		
Number of Isolates in PulseNet	**186**	**22**	**14**	**6**	**10**	**37**	**1**
U.S. per capita food availability (Pounds per capita per year)****	**62.4**	**46.5**	**73.6**	**20.6**	**10.1**	**16.5**	**31.4**
U.S. per capita food availability (Pounds per capita per year per isolate)	**0.34**	**2.11**	**5.26**	**3.43**	**1.01**	**0.45**	**31.40**

*No exposure or consumption information available for equine, reptile, or other sources
**Only Plant items that were considered for data include canteloupe and tomatoes
***Dairy products include milk and cheese
****Source: http://www.ers.usda.gov/Data/FoodConsumption/FoodAvailQueriable.aspx#midForm

Attribution analysis based on PFGE

PFGE attribution analysis is performed using three levels of clustering: 1) considering three major clusters; 2) considering major clusters and sub-clusters; 3) considering sub-clusters with no non-human isolates of unknown source.

Method 1: Attribution analysis considering three major clusters

To calculate the food commodity attribution based on PFGE results, the next step required determining the amount of food per capita attributed to each cluster. The results of the calculations are shown in Table 7. In order to calculate amount of food per capita attributed to isolates of Cluster I, the number of isolates is multiplied by pounds per capita per year per isolate for each commodity. The sum of all commodities represents the total amount of food per capita attributed to isolates of the cluster. For example, to calculate the amount of food per capita attributed to isolates of Cluster I, multiply each commodity's isolates to food per capita per year per isolate of each commodity [(6*0.34)=2.01], and sum the numbers to get the final amount [54.75]. 54.75 represents amount of food per capita attributed to isolates that are in Cluster I. The same steps are repeated for clusters II and III.

Calculation steps to figure out numbers of illnesses attributed to food commodity in Cluster I are shown in Table 8. In order to get the number of illnesses attributed to beef in Cluster I, food per capita per year per isolate of beef was divided by the sum of food per capita for Cluster I and multiplied by the amount by number of human illnesses were in cluster I; i.e. [(2.01/54.75)/5642=207].

The actual number of human illnesses and respective percent attribution for each food commodity for Clusters I, II, and III is displayed in Table 9. This method shows that approximately 1,058 human illnesses of the total 9,445 (11.3%) caused by *Salmonella* Newport can be attributed to beef.

Table 7: Food per capita per cluster calculation for three main clusters based on food commodities								
	Beef	**Pork**	**Poultry**	**Plant**		**Sea**	**Dairy**	**Food per capita for clusters (lbs)**
				Tomatoes	**Cantaloupes**			
Cluster I: Number of Isolates	6	1	3	6	10	10	0	
Amount of food consumed (lbs)	(6*0.34)= 2.01	(1*2.11)= 2.11	(3*5.26)= 15.77	(6*3.43)= 20.40	(10*1.01)= 10.01	(10*0.45)= 4.46	(0*31.40) =0	(2.01+2.11+15.77+20.40+10.01+ 4.46+0) = 54.75
Cluster II: Number of Isolates	177	20	6	0	0	4	0	
Amount of food consumed (lbs)	59.38	42.27	31.54	0.00	0.00	1.78	0.00	134.98
Cluster III: Number of Isolates	3	1	5	0	0	23	1	
Amount of food consumed (lbs)	1.01	2.11	26.29	0.00	0.00	10.26	31.40	71.06

Table 8: Calculations of Number of illnesses attributed to food commodities for three main clusters							
	Beef	**Pork**	**Poultry**	**Plant**		**Sea**	**Dairy**
				Tomatoes	**Canteloupes**		
Illnesses attributed to food commodity in Cluster I	(2.01/54.75)* 5642=207	(2.11/54.75) *5642= 217	(15.77/54.75)*5642=1624	(20.40/54.75)*5642= 2104	(10/54.75)* 5642=1030	(4.46/54.75)* 5642= 460	(0/54.75)* 5642=0

Table 9: Number of illnesses attributed to food commodities for three main clusters, n(%)								
	Beef	**Pork**	**Poultry**	**Plant**		**Sea**	**Dairy**	**Total, n (%)**
				Tomatoes	**Canteloupes**			
Illnesses attributed to food commodity in Cluster I	207 (2.2)	217 (2.3)	1624 (17.2)	2104 (22.3)	1030 (10.9)	460 (4.9)	0	5642 (59.7)
Illnesses attributed to food commodity in Cluster II	834 (8.8)	593 (6.3)	443 (4.7)	0	0	25 (0.3)	0	1895 (20.1)
Illnesses attributed to food commodity in Cluster III	27 (0.3)	57 (0.6)	706 (7.5)	0	0	275 (2.9)	843 (8.9)	1908 (20.2)
Total number of illnesses attributed to food commodities	**1058 (11.3)**	**857 (9.2)**	**2692 (29.4)**	**1996 (22.3)**	**1030 (10.9)**	**737 (8.0)**	**843 (8.9)**	**9445 (100)**

Method 2: Attribution analysis considering major clusters and sub-clusters

The second method divides the three big clusters into sub-clusters, and recalculates all the numbers. Number of illnesses attributed to food commodity in Clusters Ia, Ib, IIa, IIb, IIc, IId, IIe, IIIa, and IIIb are shown in Table 10.

Table 10: Number of illnesses attributed to food commodities for sub-clusters								
	Beef	**Pork**	**Poultry**	**Plant**		**Sea**	**Dairy**	**Total, n (%)**
				Tomatoes	**Canteloupes**			
Illnesses attributed to food commodity in Cluster Ia, n	33	0	1743	1875	0	441	0	4093
Illnesses attributed to food commodity in Cluster Ib, n	148	186	0	300	880	35	0	1549
Illnesses attributed to food commodity in Cluster I, n(%)	**181 (1.9)**	**186 (2.0)**	**1743 (18.5)**	**2175 (23.0)**	**880 (9.3)**	**476 (5.0)**	**0 (0.0)**	**5642 (59.7)**
Illnesses attributed to food commodity in Cluster IIa, n	281	172	0	0	0	0	0	453
Illnesses attributed to food commodity in Cluster IIb, n	62	28	277	0	0	24	0	390
Illnesses attributed to food commodity in Cluster IIc, n	24	31	0	0	0	0	0	55
Illnesses attributed to food commodity in Cluster IId, n	399	298	107	0	0	0	0	804
Illnesses attributed to food commodity in Cluster IIe, n	77	72	45	0	0	0	0	194
Illnesses attributed to food commodity in Cluster II, n (%)	**843 (8.9)**	**601 (6.4)**	**429 (4.5)**	**0 (0.0)**	**0 (0.0)**	**24 (0.3)**	**0 (0.0)**	**1896 (20.1)**
Illnesses attributed to food commodity in Cluster IIIa, n	0	0	0	0	0	319	0	319
Illnesses attributed to food commodity in Cluster IIIb, n	24	51	631	0	0	128	754	1588
Illnesses attributed to food commodity in Cluster III, n(%)	**24 (0.3)**	**51 (0.5)**	**631 (6.7)**	**0 (0.0)**	**0 (0.0)**	**447 (4.7)**	**754 (8.0)**	**1907 (20.2)**
Total number of illnesses attributed to food commodities, n(%)	**1048 (11.1)**	**838 (8.9)**	**2803 (29.7)**	**2175 (23.0)**	**880 (9.3)**	**947 (10.0)**	**754 (8.0)**	**9445 (100)**

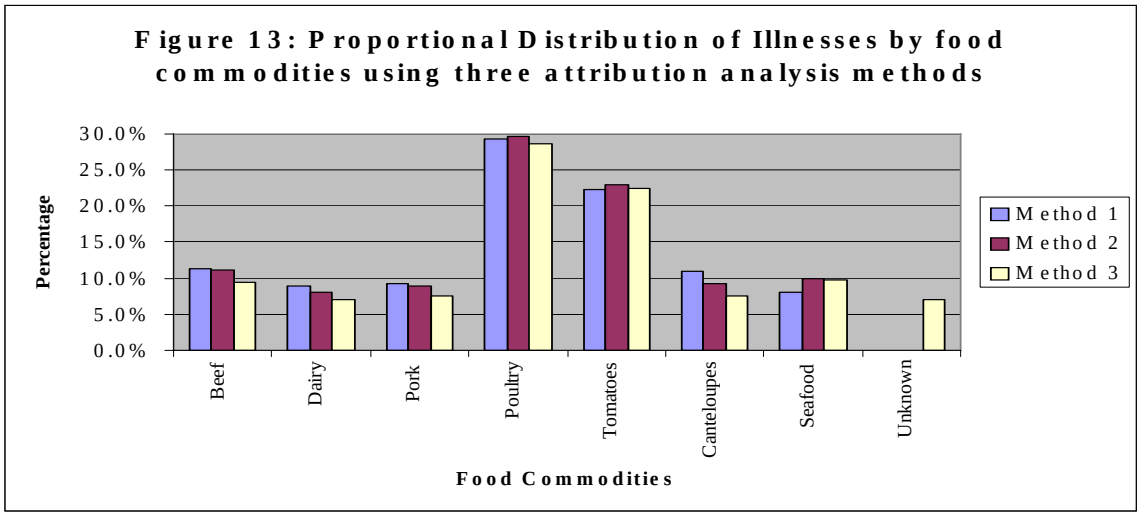
Method 3: Considering sub-clusters with no non-human isolates of unknown source

In order to bring one more level of detail into the attribution model, a third way of testing was utilized. The third method considered sub-clusters with no non-human isolates of unknown source. Isolates that clustered outside the defined clusters with non-human isolates were attributed to an unknown source. Isolates that were part of human cluster groups J, L, and O (Clusters IIIb, Ib, and IId, respectively, in Figure 12) with the least association with non-human clusters were categorized as having an unknown source. Total of 244 isolates from Group J, 280 isolates from Group L, and 149 isolates from Group O were re-categorized with this method. All unknown isolates were considered into a separate category. The number of illnesses attributed to each food commodity was recalculated, and the results are shown in Table 11.

Table 11: Number of illnesses attributed to food commodities for sub-clusters after no non-human isolates as having unknown source

	Beef	Pork	Poultry	Plant		Sea	Dairy	Unknown	Total, n (%)
				Tomatoes	Canteloupes				
Illnesses attributed to food commodity in Cluster Ib, n	33	0	1743	1875	0	441	0		4093
Illnesses attributed to food commodity in Cluster Ia, n	121	152	0	245	721	29	0		1268
Illnesses attributed to food commodity in Cluster I, n(%)	**154 (1.6)**	**152 (1.6)**	**1743 (18.5)**	**2121 (22.5)**	**721 (7.6)**	**470 (5.0)**	**0 (0.0)**		**5361 (56.8)**
Illnesses attributed to food commodity in Cluster IIa, n	281	172	0	0	0	0	0		453
Illnesses attributed to food commodity in Cluster IIb, n	62	28	277	0	0	24	0		390
Illnesses attributed to food commodity in Cluster IIc, n	24	31	0	0	0	0	0		55
Illnesses attributed to food commodity in Cluster IId, n	278	207	74	0	0	0	0		559
Illnesses attributed to food commodity in Cluster IIe, n	77	72	45	0	0	0	0		194
Illnesses attributed to food commodity in Cluster II, n (%)	**722 (7.6)**	**510 (5.4)**	**396 (4.2)**	**0 (0.0)**	**0 (0.0)**	**24 (0.3)**	**0 (0.0)**		**1652 (17.5)**
Illnesses attributed to food commodity in Cluster IIIa, n	0	0	0	0	0	319	0		319
Illnesses attributed to food commodity in Cluster IIIb, n	21	45	560	0	0	114	668		1408
Illnesses attributed to food commodity in Cluster III, n(%)	**21 (0.2)**	**45 (0.5)**	**560 (5.9)**	**0 (0.0)**	**0 (0.0)**	**433 (4.6)**	**668 (7.1)**		**1727 (18.3)**
Total number of illnesses attributed to food commodities, n(%)	**897 (9.5)**	**707 (7.5)**	**2699 (28.6)**	**2121 (22.5)**	**721 (7.6)**	**927 (9.8)**	**668 (7.1)**	**673 (7.1)**	**9445 (100)**

Figure 13 shows the proportion of attribution of illness to food commodities based on all three methods: considering major clusters, considering sub-clusters, and considering unknown category. This proportion considers all 9,445 human *Salmonella* Newport isolates submitted from 2003 to 2006.



Method 1: Considering three major clusters
Method 2: Considering major clusters and sub-clusters
Method 3: Considering sub-clusters with no non-human isolates of unknown source.

According to all three methods, the highest proportional distribution of *Salmonella* Newport illnesses was poultry, followed by tomatoes, and then dairy and beef products combined. The combined proportion of *Salmonella* Newport from beef and dairy products was 17.6% according to Method 1, 16.3% according to Method 2, and 15% according to Method 3.

Antimicrobial Sensitivity Testing Analysis

Out of 617 *Salmonella* Newport isolates tested by NARMS from 2003 to 2004, 382 isolates were submitted to the PulseNet database. Table 12 shows antimicrobial resistance patterns of human *Salmonella* Newport isolates from the U.S. from 2003-2004 based on data from NARMS. Antimicrobial agents tested included aminoglycosides (kanamycin, gentamicin, streptomycin), ampicillin, one beta-lactouse inhibitor combinations (amoxicillin-clavulanic acid), 1st generation cephalosporins (cephalothin), 3rd generation cephalosporins ceftriaxone, cephamycins (cefoxitin), folate pathway inhibitors (trimethoprim-sulfamethoxazole), phenicols (chloramphenicol), quinolones (nalidixic acid, ciprofloxacin), sulfisoxazole, and tetracycline. Isolates that were resistant to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, cephalothin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, and decreased susceptibility to ceftriaxone were classified as multi-drug resistant (MDR) AmpC.

Table 12: Antimicrobial resistance patterns of human Salmonella Newport isolates from the U.S. (2003-2004), based on data collected from NARMS

Resistance Patterns		**Frequency (%) case patients from the U.S. (n=382)**
No detected resistance		302 (79.0%)
Resistance to 1 antimicrobial agent		4 (1.0%)
Resistance to 2 antimicrobial agent		3 (0.8%)
Resistance to 3 antimicrobial agent		4 (1.0%)
Resistance to 4 antimicrobial agent		5 (1.2%)
Resistance to 5 antimicrobial agent		0 (0.0%)
At least MDR-AmpC resistant		64 (17%)
Total		**382**

Among the 64 Newport MDR-AmpC isolates, 7 (11%) met the National Committee on Clinical Laboratory Standards (NCCLS) criteria for resistance to ceftriaxone. Additionally, 15 (23%) were resistant to kanamycin, 3 (5%) were resistant to trimethoprim/sulfamethoxazole, and 2 (3%) were resistant to gentamicin. All the Newport MDR-AmpC isolates were susceptible to nalidixic acid, ciprofloxacin, and amikacin (Table 13).

Table 13: Antimicrobial sensitivity testing of Newport MDR-AmpC, compared to that of other *Salmonella* Newport isolates (2003-2004)

	NARMS	
Antimicrobial agent	**Newport MDR-AmpC n (%) resistant** (n=64)	**Other Newport n (%) resistant** (n=318)
Ampicillin	64 (100)	4 (1)
Chloramphenicol	64 (100)	3 (<1)
Streptomycin	64 (100)	7 (2)
Tetracycline	64 (100)	8 (3)
Amoxicillin/Clavulanic Acid	64 (100)	0
Cefoxitin	64 (100)	0
Ceftiofur	64 (100)	1 (<1)
Cephalothin	34 of 34 (100)	0
Sulfamethoxazole	34 of 34 (100)	4 of 114 (4)
Sulfisoxazole	30 of 30 (100)	9 of 209 (4)
Kanamycin	15 (23)	0
Ceftriaxone	7 (11)	0
Trimethoprim/Sulfamethoxazole	3 (5)	2 (<1)
Gentamicin	2 (3)	4 (1)
Ciprofloxacin	0	0
Nalidixic Acid	0	0
Amikacin	0	0

Attribution analysis based on PFGE and Antimicrobial Sensitivity Testing

Pan-susceptible

Out of 382 isolates tested by NARMS, 79% (302/382) were pan-susceptible and displayed a total of 159 different PFGE patterns. Table 14 displays the number of different pan-susceptible *Salmonella* Newport PFGE patterns by geographical regions from 2003-2004. These 159 patterns were significantly different from the Newport MDR-AmpC patterns.

Table 14: Number of *S.* Newport Pansusceptible Patterns, 2003-2004	
Northeast Central	21
Southeast Central	10
MidAtlantic	29
Mountain	10
New England	10
Pacific	12
South Atlantic	46
Northwest Central	17
Southwest Central	52

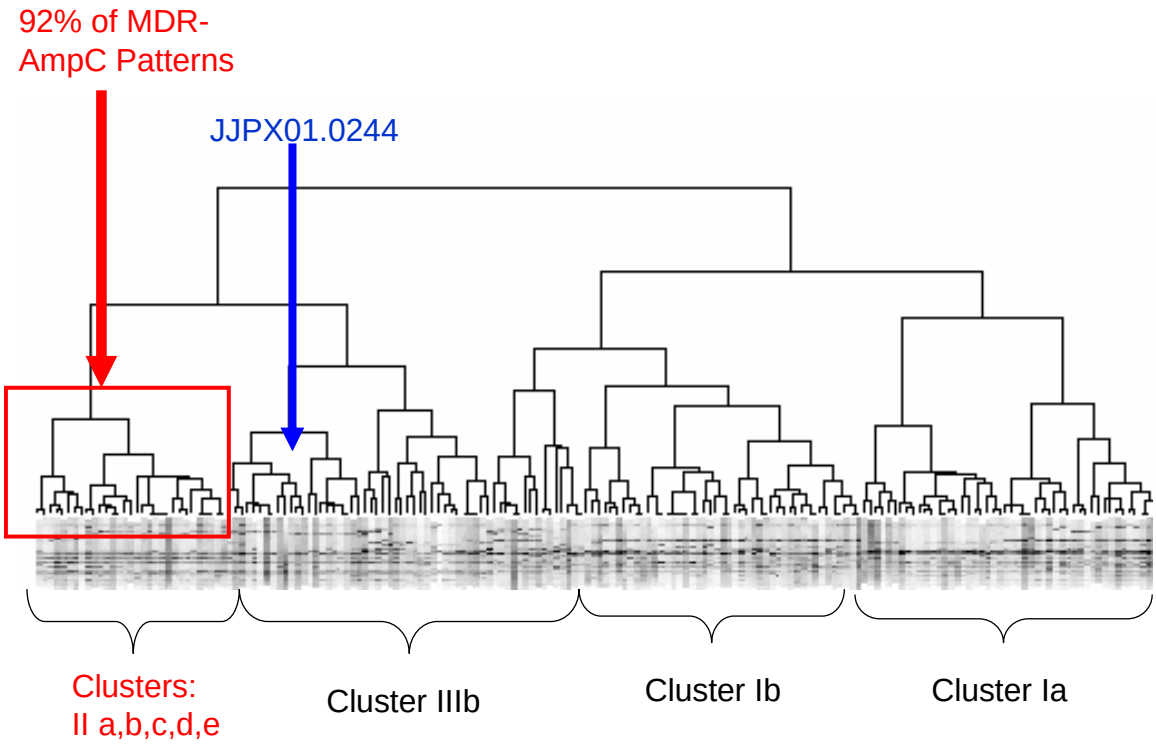
Newport MDR-AmpC

Out of 382 isolates tested by NARMS, 17% (64/382) of the isolates were identified as *Salmonella* Newport MDR-AmpC. Table 15 displays the number of different *Salmonella* Newport MDR-AmpC PFGE patterns by geographical regions from 2003-2004. For example, northeast central region has a large array (n=13) of PFGE patterns in one region.

Table 15: Number of *S.* Newport MDR-AmpC Patterns, 2003-2004	
Northeast Central	13
Southeast Central	1
MidAtlantic	7
Mountain	4
New England	5
Pacific	2
South Atlantic	1
Northwest Central	4
Southwest Central	1

Among 64 isolates, there were 24 unique PFGE patterns identified. All MDR-AmpC patterns, except for patterns JJPX01.0244 and JJPX01.1359, clustered in Cluster II a, b,c,d, and e, and they are highly related to each other. These PFGE patterns are indicated in the red box in Figure 14. Among the Newport MDR-AmpC isolates, the most prevalent PFGE patterns were JJPX01.0014 (shared by 27 (42%) of the isolates and JJPX01.0085 (shared by 5 (8%) of the isolates). All susceptible isolates clustered in Clusters Ia, Ib, and IIIb. Cluster IIIa included all imported isolates, and they have not been tested by NARMS.

Figure 14: A dendrogram representation showing the genetic relationship of *Salmonella* Newport isolates with enzyme *Xba*I



Frequencies of top 10 human and non-human patterns were calculated (Table 16), and both groups had only one PFGE pattern in common, JJPX01.0014. Pattern JJPX01.0014 is the most common *Salmonella* Newport MDR pattern in the PulseNet database in both human and non-human groups. Of the 64 isolates from humans that had Newport MDR-AmpC PFGE patterns, 27 isolates (42%) were JJPX01.0014. This pattern JJPX01.0014 had been identified in 8 out of 10 regions, including geographically distant states, such as California, New York, Washington, and Florida.

Table 16: Comparing Top Human and non-Human PFGE Patterns	
Top 10 *S*. Newport Human Patterns	**Top 10 *S*. Newport non-Human Patterns**
JJPX01.0012	JJPX01.0014
JJPX01.0014	JJPX01.0042
JJPX01.0011	JJPX01.0587
JJPX01.0030	JJPX01.0383
JJPX01.0061	JJPX01.0262
JJPX01.0025	JJPX01.0028
JJPX01.0041	JJPX01.0977
JJPX01.0010	JJPX01.0085
JJPX01.0085	JJPX01.0198
JJPX01.0372	JJPX01.0238

The total number of human isolates that were associated with 24 PFGE patterns of MDR-AmpC isolates by year are shown in Table 17. The list is organized based on decreasing pattern prevalence. The results show that out of 24 Newport MDR-AmpC patterns seen in humans, 22 patterns have been declining since 2003. Two patterns, JJPX01.0244 and JJPX01.0258, were decreasing but stopped and began to increase. The PulseNet outbreak log shows that pattern JJPX01.0258 was involved in a multi-state outbreak in 2006, but no food commodity source was identified for the cause of the outbreak. Pattern JJPX01.0244 has not been associated with an outbreak (Lockett, 2007, personal communication).

One isolate with pattern JJPX01.0244 was isolated from a poultry product in 2003. Two isolates with pattern JJPX01.0258 were isolated from beef in 2003. All of the MDR-AmpC patterns clustered in Cluster II except for pattern JJPX01.0244. Pattern JJPX01.0244 clustered with isolates of Cluster IIIb, and this is one of the two MDR-

AmpC patterns that was decreasing from 2003 to 2005 but stopped and began to increase again in 2005.

Table 17:PFGE patterns of human isolates for all MDR-AmpC Patterns, 2003-2006					
Newport MDR-AmpC Patterns	**Total Isolates n (%)**	**Year**			
		2003	**2004**	**2005**	**2006**
JJPX01.0014	**486 (34.8)**	226	103	77	80
JJPX01.0085	**151 (10.8)**	33	66	31	21
JJPX01.0238	**136 (9.7)**	60	33	28	15
JJPX01.0042	**107 (7.7)**	45	26	16	20
JJPX01.0247	**107 (7.7)**	74	22	7	4
JJPX01.0258	**64 (4.6)**	12	17	5	30
JJPX01.0383	**46 (3.3)**	10	10	18	8
JJPX01.0248	**43 (3.1)**	28	12	1	2
JJPX01.0181	**38 (2.7)**	14	13	6	5
JJPX01.0244	**36 (2.6)**	14	3	6	13
JJPX01.0254	**33 (2.4)**	18	8	6	1
JJPX01.0028	**31 (2.2)**	18	12	1	0
JJPX01.0250	**25 (1.8)**	14	8	2	1
JJPX01.0253	**21 (1.5)**	10	5	4	2
JJPX01.0279	**19 (1.4)**	1	14	4	0
JJPX01.0593	**10 (0.7)**	6	2	1	1
JJPX01.0176	**9 (0.6)**	2	5	1	1
JJPX01.0353	**9 (0.6)**	3	0	3	3
JJPX01.0204	**7 (0.5)**	4	1	1	1
JJPX01.1359	**7 (0.5)**	0	7	0	0
JJPX01.1795	**6 (0.4)**	1	5	0	0
JJPX01.1817	**3 (0.2)**	2	1	0	0
JJPX01.1398	**2 (0.1)**	0	0	2	0
JJPX01.1819	**1 (0.0)**	0	1	0	0
Total	**1397 (100)**	**595**	**374**	**220**	**208**

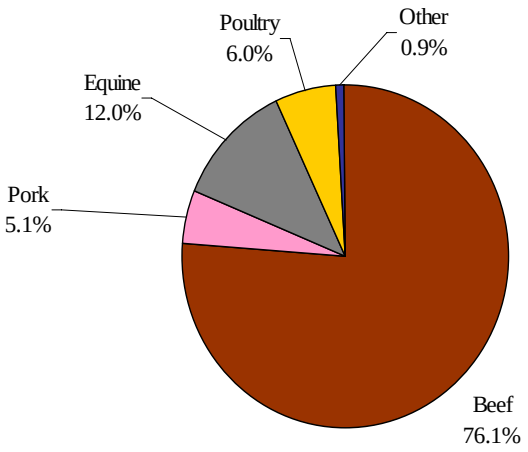
Table 18 further illustrates the number of food commodity isolates collected for 24 MDR-AmpC patterns for years 2003-2006. Out of 24 MDR-AmpC patterns, 15 patterns have been associated with non-human sources. *Salmonella* Newport patterns

JJPX01.0181, JJPX01.0248, JJPX01.0250, JJPX01.0253, JJPX01.1359, JJPX01.1398, JJPX01.1795, JJPX01.1817, and JJPX01.1819 were isolated from humans only.

Table 18: PFGE patterns of isolates collected for each food commodities for all 24 MDR-AmpC patterns for years 2003-2006					
Newport MDR-AmpC Patterns	**Total Isolates n (%)**	**2003**	**2004**	**2005**	**2006**
Soruce: Beef					
JJPX01.0014	**36 (40.4)**	22	11	1	2
JJPX01.0042	**29 (32.6)**	7	5	0	17
JJPX01.0028	**7 (7.9)**	2	2	3	0
JJPX01.0085	**6 (6.7)**	1	1	2	2
JJPX01.0383	**4 (4.5)**	0	1	2	1
JJPX01.0247	**3 (3.4)**	1	0	2	0
JJPX01.0353	**2 (2.2)**	2	0	0	0
JJPX01.0238	**1 (1.1)**	0	1	0	0
JJPX01.0258	**1 (1.1)**	1	0	0	0
Total	**89 (100)**	**36**	**21**	**10**	**22**
Source: Pork					
JJPX01.0042	**2 (33.3)**	2	0	0	0
JJPX01.0204	**2 (33.3)**	2	0	0	0
JJPX01.0014	**1 (16.7)**	0	0	1	0
JJPX01.0593	**1 (16.7)**	1	0	0	0
Total	**6 (100)**	**5**	**0**	**1**	**0**
Source: Equine					
JJPX01.0383	**9 (64.3)**	0	9	0	0
JJPX01.0028	**2 (14.3)**	0	2	0	0
JJPX01.0014	**1 (7.1)**	0	0	1	0
JJPX01.0042	**1 (7.1)**	0	1	0	0
JJPX01.0238	**1 (7.1)**	1	0	0	0
Total	**14 (100)**	**1**	**12**	**1**	**0**
Source: Poultry					
JJPX01.0238	**3 (42.3)**	0	0	3	0
JJPX01.0244	**2 (28.6)**	2	0	0	0
JJPX01.0176	**1 (14.3)**	1	0	0	0
JJPX01.0279	**1 (14.3)**	0	0	1	0
Total	**7 (100)**	**3**	**0**	**4**	**0**
Source: Other					
JJPX01.0254	**1 (100)**	1	0	0	0
Total	**1 (100)**	**1**	**0**	**0**	**0**

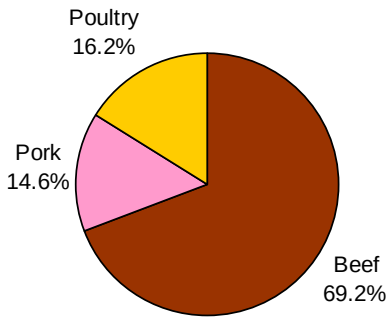
Sources of non-human isolates in the PulseNet USA *Salmonella* database with MDR-AmpC PFGE patterns can be seen in Figure 15. Over 75% of MDR-AmpC patterns isolated from 2003 to 2006 were from beef.

Figure 15: MDR-AmpC pattern distribution among non-human sources



Relative attribution of *Salmonella* Newport MDR-AmpC infections is displayed in Figure 16. The calculations performed using the same steps as attribution analysis of PFGE show that beef source causes the highest amt of MDR-AmpC *Salmonella* Newport infections, and pork and poultry are almost equal, about 15%.

Figure 16: Attribution of *Salmonella* Newport MDR-AmpC infections to food commodities



CHAPTER V

DISCUSSION

Study Significance

Salmonella is responsible for causing approximately 1.4 million illnesses per year in the U.S. (Mead, 1999), and the estimated cost of *Salmonella* illnesses in 2005 was $2.4 trillion in the U.S. (USDA-ERS, 2007). *Salmonella* serotype Newport is the third most common cause of salmonellosis in the U.S. over the past 10 years, and causes more than 100,000 infections annually in the U.S. (Greene, 2007). Antimicrobial agents have been widely used in human and animal populations to control infectious diseases caused by this bacteria, and this has led to the emergence of MDR *Salmonella* strains in animals and humans (Zhao, 2003). The emergence of MDR strains coupled with an increase of *Salmonella* Newport prevalence is a serious public health problem across the U.S. In an effort to identify the potential food commodities that are responsible for causing these illnesses, this study performs microbiological attribution analysis and focuses on determining the relative contribution of different food sources to infections caused by this pathogen. The two methods used to perform attribution analysis including microbial subtyping by PFGE and antimicrobial sensitivity testing. This study is the first attempt to use this kind of data for attribution analysis of salmonellosis in the U.S.

Important Study Findings

It was hypothesized that 1) DNA fingerprint patterns of isolates collected from non-human sources will correlate and cluster with isolates collected from humans and is

useful for attribution analysis, and 2) MDR will be restricted to isolates from animal sources, and it will not be present in isolates from produce sources. The study findings discussed below illustrate that both of these assumptions are proven to be true in the data analyzed.

Results from this study show that since 2003, *Salmonella* Newport strains submitted to the PulseNet database has been gradually increasing in the U.S. There is a wide array of sources attributed to *Salmonella* Newport infections; including beef, seafood, plant food, equine, pork, poultry, reptile, dairy, and a few other products. Beef isolates ranked the highest for non-human isolates submitted to the PulseNet database. After beef, the order is seafood, plant food, equine, pork, poultry, reptile, and dairy. While the number of isolates in the study relies on the ability of participating public health laboratories to submit isolates, these numbers do show some interesting trends. This study shows that the number of isolates received from beef, dairy, and reptile sources have been consistent from 2003 to 2006. Additionally, isolates received from a plant and seafood sources have been gradually increasing, and isolates received from pork source has been decreasing. The number of isolates received from poultry went from zero isolates in 2003 to eight in 2005, and then decreased again to one isolate in 2006, while the number of isolates from equine went from three in 2003 to 17 in 2004 and down to zero in 2006. The increase in equine and poultry isolates during 2004 and 2005 could be due to an increase in testing of horses and poultry due to the emergence of the West Nile Virus, which was known to be transmitted by these sources (CDC-MMWR, 2006).

The further analysis of data required performing the cluster analysis of the non-human, and this analysis revealed important findings. The dendrogram created a genetic tree and placed the isolates into separate clusters according to their relationship to each other. Cluster I included isolates from all sources except dairy. The largest contributor of Cluster I was plant isolates, followed by equine isolates. Furthermore, even though beef isolates were submitted from all across the U.S., over 95% of beef isolates clustered in Cluster II. In fact, all the isolates in Cluster II were isolated from domestic U.S. products, including over 90% of pork and 60% of equine isolates. Over 60% of seafood isolates clustered in Cluster III. All isolates that were part of Cluster IIIa, including approximately 30% (11/37) of the seafood isolates, were from imported food items. Poultry isolates were divided among all three clusters. The data emphasizes the importance of understanding that *Salmonella* Newport is prevalent in an array of food items, including seafood, animals and produce. These findings show that prevalence of *Salmonella* Newport is a major public health concern because these products get consumed daily and finding the source of infections can be very challenging because of the bacteria's ability to manifest in a variety of products. Therefore, understanding the genetic relationship, by comparing PFGE patterns, of the pathogen can help link the pathogen to its contribution source and help alleviate the problem that is caused by the organism.

Findings from this study show that in humans, *Salmonella* Newport was responsible for causing illness for all age ranges. Isolates collected from humans in the PulseNet database ranged from 1 day old to 99 years old. The data shows that infections are evenly distributed between the sexes. Infection was geographically distributed across

the nation; however, the South Atlantic region had the highest amount of *Salmonella* infections. The South Atlantic region includes Delaware, District of Columbia, Florida, Georgia, Maryland, North Carolina, Puerto Rico, South Carolina, Virginia, and West Virginia. The New England region has the least amount of infections and includes Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont.

In this study, the dendrogram of human and non-human isolates revealed many interesting results. Findings showed that most human clusters had strong association with non-human clusters in a dendrogram. Only three human clusters, J, L, and O, had little or no association to non-human clusters. Isolates in human cluster group L clustered outside the defined clusters with non-human isolates, and due to that reason attributed to an unknown source. Isolates that were part of human clusters J and K showed very little association to their respective non-human clusters, and for that reason those isolates were categorized as unknown in Method 3 of attribution analysis. Testing of the attribution model with three methods showed similar results. For instance, the combined proportion of *Salmonella* Newport from beef and dairy products was 17.6% according to Method 1, 16.3% according to Method 2, and15% according to Method 3. These results show that Method 1 considers three major clusters for attribution analysis provides broad level results of attribution. Method 2 considers major clusters and sub-clusters, and provides number of illnesses attributed to food commodities for each sub-cluster. Method 3 brings one more level of detail into the attribution model, and considers sub-clusters with no non-human isolates of unknown source.

The proportional distribution of illnesses by food commodities using three attribution analysis methods showed that the highest proportional distribution of

Salmonella Newport illnesses was poultry, followed by tomatoes, and then beef and dairty products combined. There were only 14 poultry isolates submitted from 2003 to 2006, and the number of illnesses attributable to the poultry isolates and their PFGE patterns was approximately 25%. This result indicates that efforts need to be made, perhaps at the farm level, to reduce the prevalence of *Salmonella* in poultry and to reduce transmission of Newport. Even though beef and dairy isolates (n=187) were the highest non-human *Salmonella* Newport isolates submitted to PulseNet from 2003 to 2006, the number of illnesses attributable to the sources' patterns was approximately 17%. Furthermore, only 16 tomato and 10 cantaloupe isolates were received during the study period; however, the number of human illnesses attributed to these two sources was approximately 18% and 9%, respectively. Hence, even though the numbers of beef isolates was high, the number of human illnesses attributed to beef was low when compared to plant foods, more specifically tomatoes and cantaloupes. The results of this study rely heavily on the number of non-human isolates collected, tested, and submitted to the PulseNet database to understand genetic relatedness of the non-human sources. The finding of the data emphasizes the importance of reviewing DNA patterns of each food commodity and comparing the patterns to human isolates in order to attribute number of illnesses to any specific food commodity.

Antimicrobial sensitivity testing data showed that approximately 79% of *Salmonella* Newport isolates had no detected resistance, and approximately 17% of the isolates showed MDR-AmpC resistance. Newport MDR-AmpC isolates were resistant to nine different antimicrobials: ampicillin, amoxicillin-clavulanic acid, cefoxitin, cephalothin, ceftiofur, chloramphenicol, streptomycin, sulfamethoxazole, and

tetracycline. Results of attribution analysis using PFGE and antimicrobial sensitivity data showed that even though *Salmonella* Newport strains submitted to the PulseNet database has been gradually increasing in the U.S., the number of *Salmonella* Newport MDR-AmpC strains have been, for the most part, steadily decreasing, while the number of pan-susceptible patterns has been increasing since 2003. In 2003, 24.8% (number of human Newport MDR-AmpC pattern/total human isolates *Salmonella* Newport submitted for the year = 595/2398) of all *Salmonella* Newport strains were resistant to nine antimicrobials that are considered as MDR-AmpC. This number has decreased to 6.9% (208/3004) in 2006. There were only two MDR-AmpC patterns, JJPX01.0258 and JJPX01.0244, that initially showed a drop compared to 2003 data, but then started to rise again. An epidemiological investigation in 2006 showed that pattern JJPX01.0258 was involved in an outbreak on the West coast of the U.S., which led to an increase in the number of isolates submitted with that pattern (Lockett, 2007, personal communication). No food source was attributed to the cause of the outbreak. There was no outbreak data available to describe the increase of isolates with PFGE pattern JJPX01.0244.

Results from this study demonstrate problems facing the U.S., more specifically the young, the old, and people with an underlying immunosuppressive condition. Though MDR-AmpC patterns have been decreasing for the past two to three years, research by Devasia, et al, has showed that patients that have been infected with *Salmonella* Newport due to MDR-AmpC strains of bacteria tend to have more severe illness compared to patients with pan-susceptible strain bacterial infections (Devasia, 2005). In an event where an outbreak occurs due to a MDR-AmpC pattern, such as the

outbreak caused by pattern JJPX01.0258, severity of the health problems can be detrimental to the community and control of the problem can be challenging.

In this study, beef isolates were highly associated with MDR-AmpC pattern, JJPX01.0014. PFGE pattern JJPX01.0014 is listed as the most common non-human and second most common human pattern submitted to the PulseNet database. Pattern JJPX01.0014 is the most common *Salmonella* Newport MDR-AmpC pattern submitted to the PulseNet database. The majority of isolates with pattern JJPX01.0014 was attributed to beef.

In an effort to determine the attribution source of infections, PFGE was used to compare the number of illnesses attributed to beef and dairy products to the number of patterns attributable to Newport MDR-AmpC. The reason for this comparison is that most non-human MDR-AmpC patterns (77%=89/116) are derived from either beef or dairy products. PFGE attribution analysis was performed three different ways in this study showed that approximately 17% of human illnesses were attributable to beef and dairy products. The study also found that 17% (64/382) of *Salmonella* Newport isolates tested by NARMS are MDR-AmpC. All MDR-AmpC strains of *Salmonella* Newport were clustered in Cluster II. In fact, over 95% of beef isolates clustered in Cluster II, and all isolates in Cluster II were isolated from U.S. products, including over 90% of pork and 60% of equine isolates. Findings from this study showed that no plant or sea products were attributed to MDR-AmpC strains of *Salmonella* Newport.

The findings of study also show that there are several MDR-AmpC Newport patterns collected from humans during from 2003 to 2006 that have no non-human PFGE patterns matching the human isolates. This finding implicates that there are high risk

sources present in the environment, water, or food that are dangerous to human health, but these sources have not yet been identified. The overuse of antimicrobials may provide selective pressure for the spread of Newport MDR-AmpC to humans through these unknown sources and known sources, such as beef and poultry. Hence, efforts to promote the appropriate use of antimicrobials followed by the surveillance of these antimicrobials are necessary for the prevention and control of MDR pathogens.

Public Health Implications

One of the essential functions of public health is to "diagnose and investigate health problems and health hazards in the community" (CDC-NPHPSP, 2007). In order to investigate, prevent, and control health hazards caused by foodborne bacteria, Healthy People 2010 has listed food safety as one of the priorities of Healthy People 2010 initiative. The two objectives of this initiative are to reduce infections caused by key foodborne pathogens and to reduce outbreaks of infections caused by key foodborne bacteria (Healthy People 2010, 2000).

Surveillance, timely diagnosis, effective disease control measures and public education are necessary components of effective programs for detection and prevention of zoonotic disease in all species. Controlling Newport MDR-AmpC requires public health initiatives directed at beef industry and poultry farms, including enhanced pathogen surveillance from farm to table additional research on transmission mechanisms. This study highlights the importance of veterinarians to develop alternate non-antibiotic treatments and management strategies that can be applied to diseased or suspected diseased animals because excessive use of antibiotics has a great impact on public health. The use of antimicrobials agents creates a selective pressure that facilitates

dissemination of MDR *Salmonella* strains. Therefore, reducing unnecessary use of antimicrobials agents may help to limit the spread of MDR strains.

Additionally, control of the bacteria in fruits and vegetables requires agricultural industry to follow available guidelines for good manufacturing practices and good agricultural practices when harvesting produce. Current guidelines state that water should be suitable for its intended use. Guidelines need to be designed to ensure that all water used for agricultural purposes meet potable drinking water standards.

This study has an impact on the public health because it shows that subtyping of bacteria and attribution analysis are important to at least indicated which food commodity may be involved in causing infections and to guide outbreak detection. For instance, during an outbreak, if an isolate from the source of the outbreak is not present in the database, a PFGE match of human isolate to food commodity may at least indicate which food commodity is involved. This information can be important to the epidemiologists when they generate hypotheses about the source of the outbreak.

In order to reduce infections, it is necessary to understand the cause of the illness. This study successfully used microbial attribution analysis to understand the contribution of food commodity of human illness caused by *Salmonella* Newport. Once the illness contribution has been identified, prevention measurements can be taken to alleviate and control human illnesses caused by the specific commodity.

Study Limitations

It is important to discuss the limitations involved in the study. The first limitation is that with the use of PFGE as the microbial subtyping method, attribution is made at the

reservoir level. This method does not allow investigating different pathways through which the pathogen can be transmitted. A comparison of *Salmonella* Newport isolated from animal and plant food with isolates from humans makes it possible to produce estimates of the number of human cases attributable to sources.

Attribution analysis in this study required comparison of the number of reported human isolates caused by *Salmonella* Newport with the distribution of the *Salmonella* isolated from various food sources. This method required a systematic "farm-to-table" surveillance with data collection from representative sources, such as beef or plant sources. The second limitation is that the results of this study rely heavily on the number of non-human isolates collected, tested, and submitted to the PulseNet database to understand genetic relatedness of the non-human sources as well as to determine pounds per isolate to attribute human infection to specific sources. The PulseNet database mirrors the surveillance in the states, and sampling of isolates varies from state to state. Therefore, if non-human isolates do not represent all the tested isolates, the results of the study will not fully represent the actual attribution amount and impact the results. Since over 70 U.S. public health laboratories and regulatory agencies regularly submit isolates to the PulseNet database (Gerner-Smidt, 2006), this study assumes isolates included in this study represent the national trend of infections. This is a limitation because there is only one dairy isolate in the study, but literature sources show that dairy is a major source of *Salmonella* Newport infections (Fontaine, 1978; Holmberg, 1984; Spika, 1987; Zhao 2003), so there should be more than one dairy isolate in the database. This study is based on non-human isolates submitted by public health laboratories and federal agencies. USDA-FSIS and FDA-CVM isolates are from retail food studies only, which are

collected from ten FoodNet sites (CDC-FoodNet, 2007). Furthermore, not all federal agency collected isolates are submitted to PulseNet. Isolates collected from raw meat products by USDA-FSIS for hazard analysis and critical control point regulations are sent to USDA VetNet for laboratory testing, and data is analyzed and stored by USDA VetNet. USDA VetNet is a network similar to PulseNet that was created by USDA-ARS, and its purpose is to serve food and veterinary laboratories in the U.S. The objective of USDA VetNet is to determine PFGE profiles of foodborne pathogens isolated in food and agricultural surveillance projects. Future attribution analysis studies should compare USDA VetNet and PulseNet PFGE patterns, and use the comparative data for surveillance and investigation of foodborne illness outbreaks. Due to the lack of a Memorandum of Understanding between VetNet and PulseNet, data could not be shared between the two federal agencies.

Another limitation is that PulseNet data is strongly bias towards human isolates in the national database. Additionally, the nonhuman isolates are not a random sample of the different food commodities. Isolates collected from nonhuman sources are a mix of isolates strongly biased towards outbreak investigations and specific projects; therefore, these isolates do represent the actual prevalence of *Salmonella* Newport in nonhuman sources.

Additional limitation of the study is that commodities for which food consumption or exposure data was not available are not included in attribution analysis. For example, the result for plants only focuses on cantaloupe and tomatoes for two reasons. These two food commodities only account for 16/31 (52%) of plant isolates. Other plant food items that were contaminated with *Salmonella* Newport included sesame

seeds, pumpkin seeds, coriander, thyme, red chili powder, and black pepper. All of these items tend to be either garnishment or not main ingredients of a dish, which explains why it is difficult to measure the actual amount of consumption data for them. Furthermore, this study only reviewed consumption of raw tomatoes, and excluded canned tomatoes because there is no report showing that canned tomatoes can be contaminated by *Salmonella*. Most organisms are killed in the extensive canning and packing process. Additionally, there were 27 equine and five reptile associated *Salmonella* Newport isolates submitted to PulseNet from 2003 to 2006. All 32 of these isolates and their patterns were excluded from attribution analysis because there was no exposure information available for equine or reptiles. There is a need to study the impact of equine and reptile exposure to humans in order to determine the burden of equine and reptiles on human salmonellosis.

The method of cluster analysis and determining genetic relatedness in the study was done by creation of a dendrogram. The limitation is that PFGE method does not always provide phylogenetic relevant information; hence, a dendrogram can include some patterns in a cluster that may have a completely different evolutionary origin than others. It was assumed in this study that PFGE can be used to determine genetic relatedness. The study used the most universally applied clustering methods, Ward and UPGMA, to generate hierarchical relatedness between isolates by grouping them in a dendrogram or tree. Once the tree was generated, robust clusters that existed in both methods were used to determine genetic relatedness and forming Clusters I, II, III and their sub-clusters.

Recommendations

It is recommended that to validate results using a larger representative sample of nonhuman isolates from other data sources. For example, collaborate with USDA VetNet to obtain isolates that are collected from raw meat products to understand and confirm attribution results of this study and to improve future attribution analysis. Attribution analysis using the methods provided in this study relies on number of isolates received of each food commodity to obtain illnesses attributed to the commodities. It is important to note that the collaboration between USDA VetNet and PulseNet will only provide isolates collected from pork, poultry, beef and dairy products. FDA is responsible for collecting plant and seafood isolates, and for that reason a number of strains received for plants and seafood will not change due to the collaboration between USDA VetNet and PulseNet.

The second recommendation of this paper is to conduct more research to understand all the contributing sources of *Salmonella* Newport infections. There might be important sources present in the world that have not been tested by PulseNet and not included in this study. Hence, additional research can provide information regarding sources that have not been accounted for causing human illnesses.

The third recommendation of this paper is to analyze 2005 to 2007 NARMS data and PulseNet data to monitor the effects of antimicrobials on *Salmonella* Newport as well as to determine if there are new emerging *Salmonella* Newport MDR patterns. There were nine MDR-AmpC *Salmonella* Newport patterns in the current study that were isolated from humans only. It is important to determine the source of these patterns in

order to determine effective preventive measurements in case there is an outbreak of an MDR pattern.

The fourth recommendation is to perform attribution analysis on other *Salmonella* serotypes to understand the prevalence and trend of MDR in *Salmonella* as well as to confirm there is a true decline of MDR patterns across all *Salmonella* infections. According to the current study, illnesses caused by MDR-AmpC Newport patterns, for the most part, have been declining in the U.S.; however, this study only focuses on one serotype. If only MDR *Salmonella* Newport patterns are decreasing, then the focus needs to remain on the prevention of overall MDR *Salmonella* and their bacterial strains.

The fifth recommendation is to continue monitoring and researching antimicrobial sensitivity of *Salmonella* Newport infections because MDR-AmpC patterns of *Salmonella* Newport are still a public health hazard and can cause severe public health problems during outbreaks. Furthermore, it is necessary to monitor if MDR patterns that are seen in food commodities are not being transferred to humans via the food chain. The data obtained from such monitoring can be used to implement necessary policy changes that can impact antimicrobials used in animals that are used for food.

The sixth recommendation of the study is to analyze the geographic distribution of isolates to understand the trends in prevalence of the bacteria. One of the theories for higher prevalence of *Salmonella* Newport in some states compared to others was explored by Karon and colleagues. The study done by Karon explored if human infections due to MDR *Salmonella* Newport is higher in major dairy states, more specifically Wisconsin, since studies have suggested that dairy cattle are a major reservoir for MDR *Salmonella* Newport in the U.S. The results from the study showed

that compared to patients with pan-susceptible infections, patients with Newport MDR-AmpC infections were more likely to report contact with cattle, farms, or unpasteurized milk (Karon, 2007). Understanding of such trends can help focus preventive measure in the states with the highest number of illnesses attributed to a specific cause.

Lastly, one more recommendation of the study is to compare this study's microbiological attribution analysis results with epidemiological data to confirm the attribution sources found in the study. Epidemiological data can provide information from the actual cases as well as provide an insight of different pathways through which the pathogen can be transmitted. Attribution information from both microbiological and epidemiological data can provide the cause of illness, and the information can be used to design and implement preventive measures for the infections.

Conclusions

Salmonella Newport has emerged as the third most common *Salmonella* serotype causing human salmonellosis in the U.S. (CDC-MMWR, 2002). Identifying the potential food commodities responsible for causing these illnesses can guide in developing strategies to prevent and control infections associated with *Salmonella* Newport. The first aim of this study was to determine the relative contributions of different food commodities to human infections caused by *Salmonella* Newport in the U.S. during 2003-2006. Using microbial attribution analysis methods, PFGE and antimicrobial sensitivity testing, the relative contribution of different food commodities to human illness caused by *Salmonella* Newport was determined. Poultry, tomatoes, and beef are the top three contributors of *Salmonella* Newport in humans. This study was the first

attempt to use this kind of data for attribution analysis of salmonellosis in the U.S. The results from this pilot study show that PFGE and antimicrobial sensitivity testing can be useful tools in performing attribution analysis at the national level.

The second aim of this study was to determine if MDR is restricted to isolates with particular PFGE patterns or is MDR a universal phenomenon. Approximately 79% of isolates showed no resistance and 17% showed MDR-AmpC resistance. Among the MDR-AmpC isolates, there were 24 unique PFGE patterns identified, and 42% were pattern JJPX01.0014. This pattern was identified in eight out of the ten regions, including geographically distant states. Over 75% of MDR-AmpC patterns isolated from 2003 to 2006 were from beef. MDR-AmpC strains were isolated only from non-plant sources. The results show that Newport MDR-AmpC patterns are decreasing and seem to be restricted to isolates from animal sources. Overall, this study emphasizes the importance of controlling the use of antibiotics in animals. MDR-AmpC strains are present everywhere in the U.S., and the control of these strains is necessary to decrease the burden of *Salmonella* Newport infections on public health. There is a great need to communicate findings with consumers and food industry as well as public health and regulatory agencies to develop proper preventive measures.

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