VARIATION IN RAPD-PCR PATTERNS MAY NOT BE ATTRIBUTABLE TO GENETIC DIFFERENCES AMONG *SALMONELLA* ENTERITIDIS

by

DEMETRIUS L. MATHIS

(Under the Direction of Roy D. Berghaus)

ABSTRACT

Salmonella enterica serovar Enteritidis (SE) is a major public health concern because of the foodborne illness it causes in the US every year. The source of the most recent outbreak of SE was found to be table eggs, and eggs have been a known source for this pathogen since its emergence in the 1950's. The Centers for Disease Control and Prevention (CDC) uses PFGE to identify genetically related *Salmonella* strains, but the ability of pulsed-field gel electrophoresis (PFGE) to discriminate between epidemiologically unrelated SE isolates is limited due to their clonal nature. Random amplified polymorphic DNA (RAPD) PCR has been proposed as an alternative tool for distinguishing between SE isolates, but suffers from poor reproducibility. The goal of the described research was to determine whether increasing the PCR stringency would reduce the amount of randomness in SE RAPD DNA patterns.

INDEX WORDS: *Salmonella,* Enteritidis, RAPD, Reproducibility

VARIATION IN RAPD-PCR PATTERNS MAY NOT BE ATTRIBUTABLE TO GENETIC DIFFERENCES AMONG *SALMONELLA* ENTERITIDIS

by

DEMETRIUS L. MATHIS

BS, FORT VALLEY STATE UNIVERSITY, 2006

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

MASTER OF SCIENCE

ATHENS, GEORGIA

2010

VARIATION IN RAPD-PCR PATTERNS MAY NOT BE ATTRIBUTABLE TO GENETIC DIFFERENCES AMONG *SALMONELLA* ENTERITIDIS

by

DEMETRIUS L. MATHIS

Major Professor:	Roy D. Berghaus
Committee:	John J. Maurer
	Jeanna L. Wilson

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2010

ACKNOWLEDGEMENTS

I would like to personally show my appreciation to everyone who has helped my progress in becoming a successful research scientist:

My Committee:
Dr. Roy Berghaus Dr. John Maurer Dr. Jeanna Wilson

Collaborators:
Dr. Margie D. Lee Dr. Richard K. Bramwell Dr. Mike J. Wineland
Dr. Stephen D. Thayer Dr. Kenneth S. Macklin

My committee and collaborators provided me with the challenges, resources, and knowledge that made my experience as a graduate student possible and successful. They also shared their expertise by teaching me valuable and new techniques that greatly aided me in my research and my personal growth as a research scientist.

Co-workers:
Matt Webber Ricky Zoller
Dr. Ying Cheng The Maurer Lab

These are the excellent resources in the laboratory that assisted me in completing my experiments and obtaining the knowledge to write it. Thank you all for your patience and support.

My Family - The People Behind the Scenes

Thank you all for your love and support. The home-cooked meals and the place to go when I needed to just get away and clear my head were all very special and important to me. Mom and Dad, the motivational talks to keep me focused on my objective, and there was so much more you all did and it was all crucial in helping me to focus on my objective, and I have finally reached that point.

Dr. James Mack, Fort Valley State University chemistry professor, once told me, “College is not completely about the destination it more so about the journey to get there**.” I WOULD LIKE TO PERSONALLY THANK EVERYONE I HAVE MET ON THIS JOURNEY. WHETHER YOU KNOW IT OR NOT, YOU ALL HAVE HAD SOME IMPACT ON MY SUCCESS AS BECOMING A RESEARCH SCIENTIST.**

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Salmonella

Early History

Salmonella is a rod shaped gram-negative genus of bacteria that is considered to be one of the major causes of gastroenteritis in humans (Ohl and Miller, 2001). *Salmonella* exist as intracellular pathogens in diseased animal hosts. The organisms produce hair-like projections, resembling pili, which upon contact with epithelial cells "inject" bacterial proteins into the host cell's cytoplasm (Ohl and Miller, 2001).

Salmonella was first discovered by Theobald Smith while he was working in Dr. Daniel Salmon's laboratory looking for the organism that causes hog cholera (Kass, 1987; Schultz, 2008). Working under the assumption that this organism was the cause of hog cholera, it was given the name *Salmonella Choleraesuis.* Eventually, it was recognized that *Salmonella* was not just one organism, but a group of related organisms having very similar features.

Nomenclature

Initially, *Salmonella* nomenclature was defined in such a way that each serotype was considered to be a separate species (Kauffmann, 1966). Today, each serotype is identified by its composition of O and H antigens, and its host spectrum. For example, *Salmonella* Typhimurium caused typhoid fever in mice, and *Salmonella* Choleraesuis was originally believed to cause hog cholera. Not all *Salmonella* cause specific

illnesses in animals, so some species were given names according to the location where they were isolated. For example, we have *Salmonella* Arizona, *Salmonella* Dublin, and *Salmonella* Saintpaul. If this method was still used today, there would be more than 2,500 species of *Salmonella*. Instead, results from molecular tests have shown genetic similarities grouping the serotypes of *Salmonella* into two species, *Salmonella enterica* and *Salmonella bongori* (Brenner et al., 2000). *Salmonella enterica* was also broken into six (6) subspecies: *Salmonella enterica* subspecies *enterica; Salmonella enterica* subspecies *salamae; Salmonella enterica* subspecies *arizonae; Salmonella enterica* subspecies *diarizonae; Salmonella enterica* subspecies *houtenae; Salmonella enterica* subspecies *indica*. When this system of classification was first described, scientists used the full name whenever referring to any *Salmonella* in scientific papers to be certain there was no confusion between the different *Salmonella*s. An example of this name would be *Salmonella enterica* subspecies *enterica* serotype Typhimurium. Over time this approach was acknowledged as being too cumbersome, and shorter name variations were used. The first variation is *Salmonella* serotype Typhimurium or *Salmonella* ser. Typhimurium or the second variation *Salmonella* Typhimurium or *S.* Typhimurium (Brenner et al., 2000).

Salmonella, the Pathogen

Salmonella is estimated to cause 585 human deaths in the United States each year, with 553 of these being attributable to foodborne transmission (Mead et al., 1999). In 1996, the Foodborne Diseases Active Surveillance Network (FoodNet) was implemented by the CDC. The responsibility of this network was to (1) identify new and re-emerging foodborne pathogens, (2) observe and report how frequently foodborne

disease occurred in the US, and (3) trace sources back to foods such as meats, poultry and eggs (Anonymous, 1997). In 1997, FoodNet data from seven (7) sites in the US reported *Salmonella* was the primary cause of foodborne infection leading to physician visits, hospitalizations, and even death (Anonymous, 1998). That year, cases of salmonellosis were identified and confirmed by 37,200 cultures. Of these cultures, 92% were identified from stool samples. In 1997, 1998, and 1999, FoodNet data showed that approximately one-third of total deaths from foodborne illness were caused by *Salmonella* infections (Anonymous, 1998, 1999b; Mead et al., 1999).

Although there are over 2,500 serotypes of *Salmonella*, public health concern is focused on a few serotypes such as *S.* Enteritidis and *S.* Typhimurium that are frequently associated with human illness (Anonymous, 2000; Maijala et al., 2005; Oloya et al., 2007; Sanchez et al., 2002). In 2009, CDC's FoodNet reported that of the 6,371 *Salmonella* isolates serotyped, most cases of salmonellosis in the US were caused by four serovars: S. Enteritidis (1,226); S. Typhimurium (1,024); S. Newport (772); and S. Javiana (544). Together, these four serotypes accounted for 56% of *Salmonella* infections (Anonymous, 2010c). According to CDC's Morbidity and Mortality Weekly Report, *S.* Typhimurium and *S.* Enteritidis are, and have been for the past few years, the two most commonly isolated serovars of *Salmonella* causing human illnesses (Anonymous, 2005a, 2006, 2007a, 2008c, 2009b, 2010c). Even though these two serotypes account for approximately one-third of all human infections, many other serotypes are also involved in foodborne disease outbreaks (Anonymous, 1998, 1999b, 2000, 2005a, 2006, 2007a, 2008c, 2009b, 2010c).

All *Salmonella* serotypes are considered to be potential human pathogens, but many animals, both domestic and wild, can be colonized by *Salmonella* spp. without showing any signs of illness (Oloya et al., 2007; Sanchez et al., 2002). An animal infected with *Salmonella* can become clinically ill, an inapparent carrier, or both. Since *Salmonella* colonizes the gastrointestinal tract, an infected animal can consistently or periodically excrete organisms in its feces. Contact with feces can contaminate raw foods of animal origin during production and/or slaughter (Sanchez et al., 2002).

A study in North Dakota reported on the distribution of domestic animal species from which *Salmonella* had been isolated at the NDSU Veterinary Diagnostic Laboratory between 2000 and 2005. Of these domestic animal species, 64.7% of the Salmonella isolations were from cattle, followed by 12% from sheep and 10.9% from pigs (Oloya et al., 2007). This study found that 17 *Salmonella* serotypes were involved in both human and domestic animal infection, with the most widely distributed serotype across different animal species being S. Typhimurium. Looking at specific hosts, *S.* Dublin was most frequently isolated from cattle and *S.* Arizona from sheep (Oloya et al., 2007). The reason for the increased frequency of these two latter serotypes is believed to be host adaptation.

Some *Salmonella* serotypes are host-specific (Kingsley and Baumler, 2000). While these host-specific serotypes are primarily adapted to live in a single animal species, they can occasionally colonize and infect humans and other animals. For instance, *S.* Choleraesuis is adapted to pigs (Kingsley and Baumler, 2000), and *S.* Dublin is adapted to cattle (Kingsley and Baumler, 2000; Oloya et al., 2007) and both cause disease within these animal hosts. By comparison, the incidence of human

infections with either of these serotypes is low, although both have the ability to cause severe human illness.

Host adaption implies that once an animal becomes infected with the pathogen, the pathogen is capable of causing disease and being transmitted from animal to animal within that animal population (Kingsley and Baumler, 2000). Host adapted *Salmonella* serotypes typically have a longer duration of infection in their respective host species than do non-host adapted serotypes, resulting in a higher effective reproduction rate for the infection. For example, S. Typhimurium has a shorter duration of infection in cattle than S. Dublin, resulting in a decreased probability of transmission between animals. *S.* Typhi is an example of a host adapted serotype in humans and causes typhoid fever in people (Kingsley and Baumler, 2000).

Certain *Salmonella* serotypes are associated with severe disease in different animal species. In chickens, for example, most serotypes that colonize birds are not associated with any type of disease in the birds but *S.* Pullorum and *S.* Gallinarum can cause severe illness and death (Chappell et al., 2009; Johnson et al., 1992; Zhang-Barber et al., 1999). Pullorum disease causes a decrease in egg production and growth rates in adult birds and young infected birds usually die within the first three weeks. It also causes high mortality in both young and adult birds by causing the infection, fowl typhoid (Chappell et al., 2009; Wigley et al., 2001) which can have a mortality rate of 10-100% of an infected flock. *Salmonella* Gallinarum is genetically similar to *Salmonella* Pullorum and both belong to O serogroup D1.

Even though many animals can be colonized with *Salmonella* without showing any clinical signs of disease, contact with these animals can serve as an important

source of human illness. Reptiles have been estimated to account for approximately 6% of human *Salmonella* infections in the US (Mermin et al., 2004). Turtles, more specifically small turtles, have been directly identified as a source of *Salmonella* infection in several multistate outbreaks in the United States. In the 1970's, FDA banned the sale of pet turtles less than 4 inches in diameter to reduce *Salmonell*a transmission to children, because the median age of patients in turtle-associated *Salmonella* outbreaks was 7 years (Anonymous, 2005b, 2007b, 2008a, 2010b). It has been estimated that the turtle carrier state could be as high as 90% (Chiodini and Sundberg, 1981).

Sources of Human Salmonellosis

Nontyphoidal *Salmonella* serotypes are not typically transferred efficiently from human to human, but rather are acquired by contact with an infected animal or ingestion of a contaminated food source (Kingsley and Baumler, 2000). Salmonellosis is occasionally associated with exposure to pets, reptiles, and contaminated water, but is primarily thought to be a foodborne disease (Anonymous, 1998; Mead et al., 1999).

Salmonellosis has been associated with the consumption of contaminated raw or undercooked poultry products (Byrd et al., 1999). Even though animal origin and meat products, including poultry and eggs, have been identified as the most common sources, there are still many other sources that have been identified in large outbreaks of salmonellosis (Anonymous, 2000). For example, one of the most recent large outbreaks of salmonellosis was traced back to peanut butter (Anonymous, 2009a). Also, some large outbreaks were traced back to produce, including unpasteurized

orange juice and mangos in 1999 (Anonymous, 2000). In 2004, CDC was able to trace a multi-state outbreak back to tomatoes (Anonymous, 2005a).

Several studies have reported a higher incidence of salmonellosis in humans during warmer months (Doorduyn et al., 2006; Oloya et al., 2007; Sanchez et al., 2002). Oloya (2007) suggested that domestic animals were not a direct source of this variation. Instead, other sources, vehicles or conditions played a crucial role in the increase in infections. Oloya explains this by acknowledging that in their study they observed a steep rise in human infection, but during the time period just before the increase, they noticed a reduction in domestic animal cases (Oloya et al., 2007).

Outbreaks of salmonellosis may derive from different sources in different states. For example, in Georgia during 2001, barbecue was the most common source. Alfalfa sprouts were among the most commonly implicated vehicles of salmonellosis outbreaks in California from 1996 through 1998 (Mohle-Boetani et al., 2001; Sanchez et al., 2002). Humans have also become infected with *Salmonella* from pet iguanas, snakes, and dog or cat food, but these occurrences are rarely responsible for large outbreaks (Anonymous, 2008b; Mermin et al., 1997; Mermin et al., 2004).

In tracing back human salmonellosis, animals are often a primary source. Animals could be considered a reservoir of non-typhoidal *Salmonella*. Humans are at risk of becoming infected either directly or indirectly. Direct infection would include coming into contact with animal feces, whereas indirect infection would include fecal contamination of foods.

HAACP

Public concern and regulatory pressure led the Food Safety and Inspection Service (FSIS), a part of the United States Department of Agriculture (USDA), to mandate that meat and poultry processing plants implement a Pathogen Reduction/Hazard Analysis Critical Control Point (PR/HACCP) program (Anonymous, 1996). Programs were developed to improve the overall broiler carcass quality through consecutive stages of processing (Byrd et al., 1999), and to reduce the incidence of foodborne illness (Anonymous, 1996, 2001).

In July 1996, the USDA FSIS issued the Pathogen Reduction: HACCP systems regulation. By implementing HACCP the government wanted to reduce all pathogens in poultry and meat processing facilities. Included among these pathogens is *Salmonella*, which is the leading cause of outbreak-associated gastroenteritis in the United States (Anonymous, 2010c). FSIS completed a nationwide in-plant survey of meat and poultry facilities to determine baseline *Salmonella* prevalences and set performance standards (Anonymous, 1996).

According to HACCP guidelines, there was a maximum number of *Salmonella*-positive samples allowed (12) per set (total 51) of broiler carcasses (Anonymous, 1999a). Upon implementation of the HACCP program, the following year's *Salmonella* prevalence was lower for broilers, swine, ground beef, and ground turkey. In broiler carcasses alone, pre-HACCP prevalence was 20 percent and after the first year of implementation, it was reduced to 10.9%, but these results may not be all due to HACCP implementation because the data used in the analysis was not a random sample of all domestic meat and poultry production companies (Anonymous, 1999a).

Salmonella in Poultry Meat and Eggs

CDC reports the number of laboratory-confirmed salmonellosis cases per 100,000 population to be 16.2 in 2008 and 15.2 in 2009 (Anonymous, 2009b, 2010c). Poultry is considered to be one of the most important sources of foodborne *Salmonella* infections, with one recent example being the large outbreak of *S.* Enteritidis that was traced back to table eggs produced on two Iowa farms in 2010 (Anonymous, 2010a). In one study, *Salmonella* was obtained more frequently from retail poultry (35% in broilers and 24% in turkeys) than from retail pork (16%) or beef (6%) (White et al., 2001). Other than *Salmonella* Gallinarum and *Salmonella* Pullorum, *Salmonella* infection in broilers rarely causes clinical signs, so there is usually no indication that meat from colonized broiler flocks may be contaminated (Maijala et al., 2005).

Salmonella Diagnostics for Poultry

To isolate *Salmonella* from poultry, a sample is incubated at 41.5°C in the enrichment medium tetrathionate brilliant green broth (TTBG) with iodine for 18 hours (Blankenship et al., 1993). This step is known as enrichment because the conditions enrich the growth of *Salmonella* by decreasing the growth of other microorganisms allowing *Salmonella* to flourish.

After an overnight of enrichment, 10-μl is streaked onto a selective media known as Xylose-Lysine-Tergitol 4 (XLT4) and incubated at 37°C overnight (Damon and Hajna, 1956). XLT4 is differential medium for *Salmonella*. Tergitol 4 restricts the growth of many commensal bacteria. *Salmonella* can easily be identified using XLT4 based on the growth of black colonies, which corresponds to the production of H_2S. The H_2S positive colonies are streaked for growth on a general media agar such as Luria-Bertani medium

(LB agar), Tryptic soy agar (TSA), or Triple Sugar Iron (TSI) slants overnight at 37°C. The suspect colonies are confirmed as *Salmonella* by an agglutination test using a poly-O *Salmonella*-specific antiserum (Liljebjelke et al., 2005).

Also to detect *Salmonella* from the enrichment broth, an *invA* PCR assay can be used (Liu et al., 2002). This assay detects the presence of the *invA* gene, a unique marker for the genus *Salmonella* which is located in *Salmonella* pathogenicity island 1. A positive PCR assay and a negative culture result would lead to a secondary enrichment to improve *Salmonella* recovery (Liljebjelke et al., 2005; Waltman et al., 1993; Waltman et al., 1991). In a delayed secondary enrichment, a sample from the first enrichment is added to fresh TTBG and incubated. Then it can be re-cultured onto XLT4 and BGN bi-plates to detect the presence of *Salmonella* (Liljebjelke et al., 2005).

The O serogroups can be identified for *Salmonella* isolates using standard serological typing procedures for *Salmonella* O antigens (Liljebjelke et al., 2005).

Since there are over 2,500 serotypes of *Salmonella*, in the case of an outbreak, a method must be used to identify which isolates are related to the outbreak strain. Barrett et al typed *Salmonella* genetically using pulsed field gel electrophoresis (Barrett et al., 1994). As part of this process, *Salmonella* genomic DNA is embedded within agarose and then digested with restriction enzyme XbaI for 16 – 18 hours at 37°C. Using a PFGE apparatus, DNA fragments are run in an agarose gel overnight with switch times ranging from 2 – 40 seconds (Barrett et al., 1994). Cluster relationships can be evaluated using Tenover's rules (Tenover et al., 1995).

Serotyping is a useful tool in differentiating among *Salmonella* strains, but in the case of an outbreak, it is not discriminatory enough to identify the exact source (Bailey

et al., 2001), especially for common serotypes such as *S.* Typhimurium. It has been useful in identifying how *Salmonella* spreads within an integrated poultry system, however, especially in determining whether the pathogen is temporarily causing illness or a natural part of the environment (Bailey et al., 2001; Liljebjelke et al., 2005). PFGE is an even more discriminatory tool that is useful in comparing the genetic differences among most *S. enterica* serotypes, which can be especially important when tracing *Salmonella* transmission within a commercial poultry integrator (Liljebjelke et al., 2005).

PFGE and serotyping are both useful tools in determining the source of a *Salmonella* outbreak. Past research has shown a problem with using either tool in cases where the outbreak was caused by *S*. Enteritidis (Hudson et al., 2001; Thong et al., 1995). It is not always possible to determine genetic differences using PFGE between *S*. Enteritidis outbreaks because of the clonal nature of this serotype. Random amplified polymorphic DNA (RAPD) PCR has been proposed as an alternative typing tool to discern genetic differences among *S*. Enteritidis isolates (Hudson et al., 2001).

Transmission of *Salmonella* in poultry

The United States poultry industry is a fully integrated system. Poultry companies own the parent stock, and they have their own hatcheries and processing plants. So, they control all aspects of poultry from birth to processing. There are actually three poultry industries: table egg layer chickens, broiler chickens, and turkeys (Sanchez et al., 2002).

Identifying the sources of *Salmonella* in the poultry industry is a worldwide objective. *Salmonella* can be transmitted both vertically and horizontally. Vertical transmission of *Salmonella* is the passing on of the pathogen from parent to offspring.

Horizontal transmission is when the pathogen is picked up from any other source, usually environmental or foodborne (Cox et al., 2000; Liljebjelke et al., 2005). It is important to identify the sources of *Salmonella* contamination to successfully reduce transmission of *Salmonella*. Possible sources of horizontal transmission include but are not limited to feed, hatcheries, rodents, and the environment (Liljebjelke et al., 2005).

Some *Salmonella* serotypes such as S. Enteritidis have been identified as being transmitted vertically, meaning that they are passed directly from parent to offspring. Some strains of *Salmonella* can colonize the egg, ovaries, and/or reproductive tract causing the offspring to be infected at birth (Cox et al., 2000;Timoney et al., 1989). Byrd et al. (1999) identified the presence of *Salmonella* contamination found in the hatchery ranged from 5 to 9%. *Salmonella* contamination in the hatchery could also be a possible source for horizontal transmission infecting the *Salmonella* free birds (Byrd et al., 1999).

Liljebjelke et al. (2005) reported that both horizontal and vertical transmission was observed on a commercial poultry farm in Northeast Georgia. The authors of that study suggested that both the poultry production environment and the finished product should be monitored for *Salmonella*, since there are so many possible sources of contamination within the integrated poultry production system – including the farm environment, feed, rodents, insects, hatchery, and parental lines (Cox et al., 2000; Liljebjelke et al., 2005). They also stated that control of *Salmonella* contamination would have to be implemented at all levels of the vertically integrated production system to achieve adequate control of the pathogen (Liljebjelke et al., 2005). In order to reduce or

eliminate *Salmonella* from the environment, it is necessary to prevent its introduction or re-introduction onto the farm (Liljebjelke et al., 2005; Sanchez et al., 2002).

Current Management Practices for Salmonella Control

There are many possible entry points for *Salmonella* to contaminate the poultry industry. *Salmonella* can infect day old broiler chicks in the hatchery *and it* can also be in the environment on the poultry farm (Arsenault et al., 2007; Sanchez et al., 2002). Studies have been completed in the past to identify many possible risk factors, or vehicles, in the environment. Possible risk factors identified in some studies included pest control, downtime period, manure disposal, feed, poultry house cleaning and disinfecting, flock size, flock age, breed, visitors, farm disinfection, farm surroundings, and rodent control (Arsenault et al., 2007; Chadfield et al., 2001; Fris and Bos, 1995; Mollenhorst et al., 2005).

To effectively reduce *Salmonella* infections on the farm, the source(s) must first be identified (Sanchez et al., 2002). After identifying the possible sources, management must also be considered because *Salmonella* can often be found within the farm environment (Mollenhorst et al., 2005). Past studies found some management practices to reduce *Salmonella* on the farm. For example, Arsenault et al. 2007 found locking the house was associated with a lower risk of *Salmonella* infection in Canadian broiler houses (Arsenault et al., 2007). Biosecurity and feed and drinking water management may also reduce *Salmonella* colonization (Sanchez et al., 2002).

Vaccination is another method used to reduce and/or eliminate *Salmonella* infections in poultry. Studies have been completed using live attenuated and killed, low and high dosages, orally, and parenteral injections to determine which methods have

the best effects on reducing the pathogen (Curtiss and Hassan, 1996; Curtiss et al., 1993; Zhang-Barber et al., 1999). A study in Northeast Georgia compared vaccinating and non-vaccinating companies and found a decrease in *Salmonella* prevalence in the company that vaccinated broiler-breeder birds (Dorea et al., 2010). Other studies have also indicated vaccination can reduce *Salmonella* prevalence (Curtiss and Hassan, 1996; Curtiss et al., 1993; Gast et al., 1993; Gast et al., 1992; Hassan and Curtiss, 1994, 1996, 1997).

Salmonella control programs in the U.S. and Europe

The Finnish *Salmonella* Control Programme (FSCP) was given the responsibility of decreasing or eliminating *Salmonella* in poultry, cattle and swine (Maijala et al., 2005). As part of the Finnish program, broilers, turkeys, and egg-producing hens are examined at all production stages including at the hatchery (Maijala et al., 2005). Maijala et al. (2005) completed a risk assessment study to estimate the effect of the FSCP on human *Salmonella* infections. They concluded that the combination of two program components, the heat treatment of meat from infected broiler flocks and the removal of infected breeder flocks, was effective in reducing the incidence of salmonellosis in humans. They also noted that either approach alone would decrease *Salmonella* incidence, but a greater effect was expected when both were done together (Maijala et al., 2005).

Programs have been established in Denmark that improve biosecurity, cleaning and disinfecting, and feed to reduce contamination with *Salmonella* in the poultry industry. Also to reduce animal contamination, infected broiler breeder flocks are depopulated. Chadfield (2001) states that human salmonellosis is reflective of the

prevalence of the source of contamination. So, a reduction in the prevalence of *Salmonella* contaminated chicken would be expected to be associated with a reduction in the risk of human illness (Chadfield et al., 2001; Maijala et al., 2005). This has not been the experience in the U.S., however, where *Salmonella* prevalences in broiler carcasses have decreased substantially in recent years without a corresponding decrease in human cases (Anonymous, 2010c).

In the United States, the National Poultry Improvement Plan (NPIP) was implemented in the 1930s with the responsibility of eliminating *Salmonella* Pullorum from the commercial poultry industries. Today, NPIP concerns itself with certifying that flocks are still free of *Salmonella* Pullorum, *Salmonella* Gallinarum, and *Salmonella* Enteritidis, as well as other non-*Salmonella* pathogens (Rhorer, 2010).

Salmonella infection is prevalent in many food animal production units. Biosecurity, rodent control, and feed and drinking water management should be included in the control of *Salmonella* in food animals. By reducing the amount of *Salmonella* in the animals' environment, it will reduce the opportunities for *Salmonella* to re-infect the animals or poultry on the farm.

References

Anonymous, 1996, Pathogen Reduction; Hazard Analysis Critical Control Point (HACCP) Systems; Final Rule. Federal Register 61, 185.

Anonymous, 1997, Foodborne Diseases Active Surveillance Network (FoodNet). Emerg Infect Dis 3, 581-583.

Anonymous, 1998, From the Centers for Disease Control and Prevention. Incidence of foodborne illnesses--FoodNet, 1997. JAMA 280, 1651-1652.

Anonymous, 1999a, HACCP Implementation: First Year Salmonella Test Results. FSIS US Department of Agriculture. Washington DC. [Online] http://www.fsis.usda.gov/OPHS/haccp/salmdata.htm.

Anonymous, 1999b, Incidence of foodborne illnesses: preliminary data from the Foodborne Diseases Active Surveillance Network (FoodNet)--United States, 1998. MMWR Morb Mortal Wkly Rep 48, 189-194.

Anonymous, 2000, From the Centers for Disease Control and Prevention. Preliminary Foodnet data on the incidence of foodborne illnesses--selected sites, United States, 1999. JAMA 283, 1818-1819.

Anonymous, 2001, From the Centers for Disease Control and Prevention. Preliminary FoodNet data on the incidence of foodborne illnesses--selected sites, United States, 2000. JAMA 285, 2071-2073.

Anonymous, 2005a, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 sites, United States, 2004. MMWR Morb Mortal Wkly Rep 54, 352-356.

Anonymous, 2005b, Salmonellosis associated with pet turtles--Wisconsin and Wyoming, 2004. MMWR Morb Mortal Wkly Rep 54, 223-226.

Anonymous, 2006, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 States, United States, 2005. MMWR Morb Mortal Wkly Rep 55, 392-395.

Anonymous, 2007a, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2006. MMWR Morb Mortal Wkly Rep 56, 336-339.

Anonymous, 2007b, Turtle-associated salmonellosis in humans--United States, 2006-2007. MMWR Morb Mortal Wkly Rep 56, 649-652.

Anonymous, 2008a, Multistate outbreak of human Salmonella infections associated with exposure to turtles--United States, 2007-2008. MMWR Morb Mortal Wkly Rep 57, 69-72.

Anonymous, 2008b, Multistate outbreak of human Salmonella infections caused by contaminated dry dog food--United States, 2006-2007. MMWR Morb Mortal Wkly Rep 57, 521-524.

Anonymous, 2008c, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2007. MMWR Morb Mortal Wkly Rep 57, 366-370.

Anonymous, 2009a, Multistate outbreak of Salmonella infections associated with peanut butter and peanut butter-containing products--United States, 2008-2009. MMWR Morb Mortal Wkly Rep 58, 85-90.

Anonymous, 2009b, Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2008. MMWR Morb Mortal Wkly Rep 58, 333-337.

Anonymous, 2010a, Investigation Update: Multistate Outbreak of Human Salmonella Enteritidis Infections Associated with Shell Eggs. Centers for Disease Control and Prevention [Online] http://www.cdc.gov/salmonella/enteritidis/.

Anonymous, 2010b, Multistate outbreak of human Salmonella typhimurium infections associated with pet turtle exposure - United States, 2008. MMWR Morb Mortal Wkly Rep 59, 191-196.

Anonymous, 2010c, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 states, 2009. MMWR Morb Mortal Wkly Rep 59, 418-422.

Arsenault, J., Letellier, A., Quessy, S., Boulianne, M., 2007, Prevalence and risk factors for Salmonella and Campylobacter spp. carcass contamination in broiler chickens slaughtered in Quebec, Canada. Journal of food protection 70, 1820-1828.

Bailey, J.S., Stern, N.J., Fedorka-Cray, P., Craven, S.E., Cox, N.A., Cosby, D.E., Ladely, S., Musgrove, M.T., 2001, Sources and movement of Salmonella through integrated poultry operations: a multistate epidemiological investigation. J Food Prot 64, 1690-1697.

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

Blankenship, L.C., Bailey, J.S., Cox, N.A., Stern, N.J., Brewer, R., Williams, O., 1993, Two-step mucosal competitive exclusion flora treatment to diminish salmonellae in commercial broiler chickens. Poult Sci 72, 1667-1672.

Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R., Swaminathan, B., 2000, Salmonella nomenclature. J Clin Microbiol 38, 2465-2467.

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

Chadfield, M., Skov, M., Christensen, J., Madsen, M., Bisgaard, M., 2001, An epidemiological study of Salmonella enterica serovar 4, 12:b:- in broiler chickens in Denmark. Vet Microbiol 82, 233-247.

Chappell, L., Kaiser, P., Barrow, P., Jones, M.A., Johnston, C., Wigley, P., 2009, The immunobiology of avian systemic salmonellosis. Vet Immunol Immunopathol 128, 53-59.

Chiodini, R.J., Sundberg, J.P., 1981, Salmonellosis in reptiles: a review. Am J Epidemiol 113, 494-499.

Cox, N.A., Berrang, M.E., Cason, J.A., 2000, Salmonella penetration of egg shells and proliferation in broiler hatching eggs--a review. Poult Sci 79, 1571-1574.

Curtiss, R., 3rd, Hassan, J.O., 1996, Nonrecombinant and recombinant avirulent Salmonella vaccines for poultry. Vet Immunol Immunopathol 54, 365-372.

Curtiss, R., 3rd, Kelly, S.M., Hassan, J.O., 1993, Live oral avirulent Salmonella vaccines. Vet Microbiol 37, 397-405.

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

Doorduyn, Y., Van Den Brandhof, W.E., Van Duynhoven, Y.T., Wannet, W.J., Van Pelt, W., 2006, Risk factors for Salmonella Enteritidis and Typhimurium (DT104 and non-DT104) infections in The Netherlands: predominant roles for raw eggs in Enteritidis and sandboxes in Typhimurium infections. Epidemiol Infect 134, 617-626.

Dorea, F.C., Cole, D.J., Hofacre, C., Zamperini, K., Mathis, D., Doyle, M.P., Lee, M.D., Maurer, J.J., 2010, Effect of Salmonella vaccination of chicken breeders on reducing carcass contamination of broiler chickens in integrated poultry operations. Appl Environ Microbiol.

Fris, C., Bos, J., 1995, A retrospective case-control study of risk factors associated with Salmonella enterica subsp. enterica serovar Enteritidis infections on Dutch broiler breeder farms. Avian Pathol 24, 255-272.

Gast, R.K., Stone, H.D., Holt, P.S., 1993, Evaluation of the efficacy of oil-emulsion bacterins for reducing fecal shedding of Salmonella enteritidis by laying hens. Avian Dis 37, 1085-1091.

Gast, R.K., Stone, H.D., Holt, P.S., Beard, C.W., 1992, Evaluation of the efficacy of an oil-emulsion bacterin for protecting chickens against Salmonella enteritidis. Avian Dis 36, 992-999.

Hassan, J.O., Curtiss, R., 3rd, 1994, Development and evaluation of an experimental vaccination program using a live avirulent Salmonella typhimurium strain to protect immunized chickens against challenge with homologous and heterologous Salmonella serotypes. Infect Immun 62, 5519-5527.

Hassan, J.O., Curtiss, R., 3rd, 1996, Effect of vaccination of hens with an avirulent strain of Salmonella typhimurium on immunity of progeny challenged with wild-Type Salmonella strains. Infect Immun 64, 938-944.

Hassan, J.O., Curtiss, R., 3rd, 1997, Efficacy of a live avirulent Salmonella typhimurium vaccine in preventing colonization and invasion of laying hens by Salmonella typhimurium and Salmonella enteritidis. Avian Dis 41, 783-791.

Hudson, C.R., Garcia, M., Gast, R.K., Maurer, J.J., 2001, Determination of close genetic relatedness of the major Salmonella enteritidis phage types by pulsed-field gel electrophoresis and DNA sequence analysis of several Salmonella virulence genes. Avian Dis 45, 875-886.

Johnson, D.C., David, M., Goldsmith, S., 1992, Epizootiological investigation of an outbreak of pullorum disease in an integrated broiler operation. Avian Dis 36, 770-775.

Kass, E.H., 1987, A brief perspective on the early history of American infectious disease epidemiology. Yale J Biol Med 60, 341-348.

Kauffmann, F., 1966, [On the history of salmonella research]. Zentralbl Bakteriol Orig 201, 44-48.

Kingsley, R.A., Baumler, A.J., 2000, Host adaptation and the emergence of infectious disease: the Salmonella paradigm. Mol Microbiol 36, 1006-1014.

Liljebjelke, K.A., Hofacre, C.L., Liu, T., White, D.G., Ayers, S., Young, S., Maurer, J.J., 2005, Vertical and horizontal transmission of salmonella within integrated broiler production system. Foodborne Pathog Dis 2, 90-102.

Liu, T., Liljebjelke, K., Bartlett, E., Hofacre, C., Sanchez, S., Maurer, J.J., 2002, Application of nested polymerase chain reaction to detection of Salmonella in poultry environment. J Food Prot 65, 1227-1232.

Maijala, R., Ranta, J., Seuna, E., Pelkonen, S., Johansson, T., 2005, A quantitative risk assessment of the public health impact of the Finnish Salmonella control program for broilers. International journal of food microbiology 102, 21-35.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999, Food-related illness and death in the United States. Emerg Infect Dis 5, 607-625.

Mermin, J., Hoar, B., Angulo, F.J., 1997, Iguanas and Salmonella marina infection in children: a reflection of the increasing incidence of reptile-associated salmonellosis in the United States. Pediatrics 99, 399-402.

Mermin, J., Hutwagner, L., Vugia, D., Shallow, S., Daily, P., Bender, J., Koehler, J., Marcus, R., Angulo, F.J., 2004, Reptiles, amphibians, and human Salmonella infection: a population-based, case-control study. Clin Infect Dis 38 Suppl 3, S253-261.

Mohle-Boetani, J.C., Farrar, J.A., Werner, S.B., Minassian, D., Bryant, R., Abbott, S., Slutsker, L., Vugia, D.J., 2001, Escherichia coli O157 and Salmonella infections associated with sprouts in California, 1996-1998. Ann Intern Med 135, 239-247.

Mollenhorst, H., van Woudenbergh, C.J., Bokkers, E.G., de Boer, I.J., 2005, Risk factors for Salmonella enteritidis infections in laying hens. Poult Sci 84, 1308-1313.

Ohl, M.E., Miller, S.I., 2001, Salmonella: a model for bacterial pathogenesis. Annu Rev Med 52, 259-274.

Oloya, J., Theis, M., Doetkott, D., Dyer, N., Gibbs, P., Khaitsa, M.L., 2007, Evaluation of Salmonella occurrence in domestic animals and humans in North Dakota (2000-2005). Foodborne Pathog Dis 4, 551-563.

Rhorer, A.R., 2010, NPIP history for control of SE in breeding flocks. Poultry Times Special to Poultry Times.

Sanchez, S., Hofacre, C.L., Lee, M.D., Maurer, J.J., Doyle, M.P., 2002, Animal sources of salmonellosis in humans. J Am Vet Med Assoc 221, 492-497.

Schultz, M., 2008, Theobald Smith. Emerging Infectious Disease 14, [Online] http://www.cdc.gov/EID/content/14/12/1939.htm.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995, Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 33, 2233-2239.

Thong, K.L., Ngeow, Y.F., Altwegg, M., Navaratnam, P., Pang, T., 1995, Molecular analysis of Salmonella enteritidis by pulsed-field gel electrophoresis and ribotyping. J Clin Microbiol 33, 1070-1074.

Timoney, J.F., Shivaprasad, H.L., Baker, R.C., Rowe, B., 1989, Egg transmission after infection of hens with Salmonella enteritidis phage type 4. Vet Rec 125, 600-601.

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

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

White, D.G., Zhao, S., Sudler, R., Ayers, S., Friedman, S., Chen, S., McDermott, P.F., McDermott, S., Wagner, D.D., Meng, J., 2001, The isolation of antibiotic-resistant salmonella from retail ground meats. N Engl J Med 345, 1147-1154.

Wigley, P., Berchieri, A., Jr., Page, K.L., Smith, A.L., Barrow, P.A., 2001, Salmonella enterica serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. Infect Immun 69, 7873-7879.

Zhang-Barber, L., Turner, A.K., Barrow, P.A., 1999, Vaccination for control of Salmonella in poultry. Vaccine 17, 2538-2545.

CHAPTER 2

VARIATION IN RAPD-PCR PATTERNS MAY NOT BE ATTRIBUTABLE TO GENETIC DIFFERENCES AMONG *SALMONELLA* ENTERITIDIS[1]

[1] Mathis, D.L., R.D. Berghaus, M.D. Lee, and J.J. Maurer. To be submitted to *Avian Diseases.*

Abstract

Salmonella Enteritidis (SE) is the leading cause of gastroenteritis associated with consumption of contaminated poultry meat and eggs. Because pulsed-field gel electrophoresis (PFGE) has limited utility in distinguishing between clonal SE isolates, random amplified polymorphic DNA (RAPD) PCR has been recommended as an alternative molecular fingerprinting tool. This study's objective was to determine if increasing PCR stringency would reduce the amount of randomness in RAPD DNA patterns. Three different methods for DNA template preparation were evaluated, and were found to provide comparable results with respect to the similarities observed with repeated analyses of the same SE isolates (n = 18, $P = 0.91$). An *in silico* PCR was performed to predict amplification products that would be expected from known SE genomic sequences when using three different RAPD typing primers (1247, 1283, and OPA4) and to determine whether any primer would be more likely to amplify variable regions. A comparison of within- and between-isolate similarities was performed using RAPD primer 1247, which was predicted by *in silico* analysis to yield the most variable size range of amplicons, especially from variable regions in the SE genome. Although the median within-isolate similarity (76.0%) was significantly greater than the median between-isolate similarity (66.7%; $P = 0.001$), even duplicate runs of the same SE isolates produced RAPD patterns that ranged in similarity between 61.5% and 100%. In conclusion, the reproducibility of RAPD-PCR was not sufficient to reliably distinguish between related and unrelated SE isolates. If RAPD-PCR is used to evaluate SE strain differences, duplicate samples of the same isolates must be analyzed to assess method variability.

Introduction

One of the most important challenges confronting the United States food supply is the number of foodborne outbreaks. Retail poultry products contaminated with *Salmonella* are a leading cause of foodborne illness and can be detrimental to public health, especially with the worldwide increase in the consumption of poultry (Olsen et al., 2001). Between the years 2000 and 2005, there was an increase in the frequency of isolation of *Salmonella* during regulatory testing in the U.S. poultry industry (Altekruse et al., 2006).

One serotype of particular concern is *Salmonella* Enteritidis (SE). SE is recognized as a dominant serovar, and in the 1980's it was linked to the consumption of grade A table eggs (Gast, 2005; Hudson et al., 2001; St Louis et al., 1988). Recently, poultry meat has also been implicated as a primary source of SE (Kimura et al., 2004). In the US, the Centers for Disease Control and Prevention's (CDC) FoodNet surveillance system found that the incidence of salmonellosis was 15.19 cases per 100,000 persons during 2009, with SE being the most frequently identified serotype, accounting for an estimated 19.2% of all human *Salmonella* isolates (Anonymous, 2010).

Pulse-field gel electrophoresis or PFGE has become the DNA fingerprinting method of choice for determining strain differences within a bacterial population and is the preferred strain-typing tool of the CDC for determining the source of many foodborne outbreaks in the United States via PulseNet. However, PFGE is unsatisfactory for typing SE because of its clonal nature (Hudson et al., 2001; Thong et al., 1995). Phage typing is an alternative method for the identification of SE strains

based on the susceptibility of an isolate to a battery of bacteriophages. There are at least 30 different SE phage types ranging from phage type (PT) 1 to 28, with the most frequently identified types in the US being PT4, PT8, and PT13 (Altekruse et al., 2006; Hogue et al., 1997). Phage typing can identify strain differences within certain serotypes, most notably S. Enteritidis and S. Typhimurium. However, this typing method alone may be insufficient in tracing back the source of an outbreak when multiple suppliers are involved (Betancor et al., 2004; Hudson et al., 2001).

A good molecular typing tool is expected to discriminate between isolates that have no epidemiological links. Random amplified polymorphic DNA (RAPD) is a polymerase chain reaction (PCR) based method for bacterial typing that involves using short, 10-mer primers and low stringency PCR conditions to produce a distinctive DNA pattern for bacterial strains. RAPD has previously been recommended as a possible alternative for DNA typing of SE isolates (Hilton and Penn, 1998; Hudson et al., 2001) although method variability, due to the low stringency conditions, has long been recognized as a limiting factor in the reproducibility of RAPD results (Tenover, Arbeit, and Goering 1997). The objectives of this study were to compare the effects of different DNA extraction methods on the reproducibility of RAPD-PCR results, and to compare the within- and between-isolate variability when using a primer that was expected to amplify DNA from variable regions within the SE genome.

Materials and Methods

Bacterial strains. All SE isolates used in this study were previously described in detail elsewhere (Hudson et al., 2001). Briefly, 18 different SE isolates with 8 different phage type classifications were included in the current study. Isolates were either of

chicken or human origin, and had first been isolated in either the United States or Scotland (Hudson et al., 2001).

DNA extraction methods. Bacteria were suspended in autoclaved de-ionized H_20 to an optical density of 1.00 at λ = 600 nm (Spectronic $20D^+$, Milton Roy Company, USA). Templates were prepared using three different methods for DNA isolation. The first method utilized a commercially available kit according to the manufacturer's directions (Wizard® Genomic DNA Purification Kit, Promega Corp., Madison, WI). The second method involved boiling the bacteria prior to performing PCR. For the boiling method, 1 mL of the cell suspension was pelleted by centrifugation at 4500x g for 2 min. The supernatant was removed, the cell pellet was re-suspended in de-ionized H_2O, and the cell suspension subsequently boiled at 100°C to release the template DNA. The boiled cell suspension was pelleted again at 4,500 g for 2 min. The supernatant was transferred to a new, sterile micro-centrifuge tube. The third method for preparing PCR template involved a whole cell extraction where cells were kept intact and not lysed until the first step of the RAPD PCR. For this procedure, 1 mL of the bacterial suspension was washed once in 100% ethanol, cells pelleted by centrifugation at 4,500x g for 2 min, washed once in PBS, and centrifuged a second time at 4,500x g. After being re-suspended in 1mL of autoclaved de-ionized H_2O, the cell suspension was diluted 1:20 and stored at -20°C until PCR was performed. The theoretical yield of DNA was calculated for the whole cell template, and the amount of DNA in the first two methods was estimated with a spot dilution test and diluted to the same concentration of DNA (2.2 *n*g/μL).

RAPD PCR. Three different primers (Molecular Genetics Instrumentation Facility, University of Georgia) previously reported to have good discriminatory power among SE strains were used: 1283 (GTTTCCGCCC), 1247 (AAGAGCCCGT), and OPA4 (AATCGGGCTG) (Hudson et al., 2001). The PCR was performed in a 50-µL volume containing 2 mM $MgCl_2$ PCR buffer (Idaho Technology Inc., Salt Lake City, UT), 0.1 mg/mL bovine serum albumin (BSA) (New England Biolabs, Ipswich, MA), 0.2 mM deoxynucleoside tri-phosphates (Roche, Indianapolis, IN), 1 µM primer 1283, 1247, or OPA4, 1 U Taq DNA polymerase (Denville Scienftific Inc., Metuchen, NJ) and 2.2 ng DNA was added for each reaction. 100 µL of mineral oil was added as an overlay to hold mix at the bottom of the tube throughout PCR amplification. Amplification was performed using an Amplitron® II Thermolyne thermocycler (Barnstead International, Dubuque, IA). The thermocyler program was set as follows. There was an initial denaturation/cell lysis step where samples were heated to 94^{o}C for 5 min. The PCR program consisted of 94^{o}C denaturation step for 1 min, an annealing step for 5 min, and final 72^{o}C extension step for 5 min, repeated for 45 cycles. The annealing temperature was set 2.5°C below the optimal Tm predicted for each primer in order to allow for similar mismatch capabilities among all typing primers used in this study. For primers 1283, 1247, and OPA4 the annealing temperature was 36°C and 34.4°C, and 33.6 respectively. A final 5 min extension was set at 72^{o}C following the PCR program. At the end of the run, 4^{o}C hold was set for each PCR program. 10 µL of PCR products were loaded in a 1.5% agarose gel with 0.2 µg/mL ethidium bromide and electrophoresed in 1x tris acetate ethylenediaminetetraacetic (EDTA) (TAE) for 1.5 hours at 80V (8.89 V/cm agarose gel). One lane on each end and in the middle of the

gel was loaded with molecular weight marker IV (100 bp ladder) (0.6 µg DNA/lane) (Roche, Indianapolis, IN). Gels were stained for 0.5 hours in 0.5 µg/mL ethidium bromide in deionized H_2O and then destained for 1 hour in deionized H_2O. An image was captured before and after staining and destaining using the Molecular Imager® Gel Doc™ XR System with QuantityOne software (Bio-Rad, Hercules, CA). For analysis of all RAPD gel images, bands were only used if they migrated between 100 and 2,642 base pairs (bp), the lowest and highest representative DNA bands in the molecular ladder.

In Silico PCR. An online tool was used to perform a computer-based PCR simulation for the evaluation of primers 1283, 1247, and OPA4 (http://insilico.ehu.es) (Bikandi et al., 2004). This tool allows the user to specify primer sequences and stringency conditions, followed by a simulated PCR utilizing genome sequence data from the NCBI website. For the current study, simulations were performed using the genomic data for *Salmonella enterica* subsp. *enterica* serovar Enteritidis str. P125109, the only fully sequenced SE isolate in the database (NCBI GenBank Accession #: NC_011294). Stringency specifications allowed for 2 bp mismatches. Mismatches were not allowed in the last 7 nucleotides of the 3' end for primers 1283 and OPA4, and they were not allowed in the last 6 nucleotides of the 3' end for primer 1247. Maximum band length was set to 2,000 nucleotides. This procedure identified areas of the SE genome expected to be amplified for each RAPD typing primer, and provided the size and sequence of amplified regions. The sequence given for each theoretical band was identified using the BLAST algorithm (www.ncbi.nlm.nih.gov) to determine whether they were located in conserved or variable regions.

Data Analysis. Bionumerics software (Applied-Maths Inc., Austin, TX) was used to compare and analyze banding patterns. All images were input as gray-scaled tiff images. A molecular weight marker (MW14) was used for the reference lanes. Bands were identified in the software and labeled relative to the position of reference lanes. The program calculated dice coefficients to estimate the similarities of isolates by examining DNA band numbers and size. Friedman's test was used to compare the within-isolate similarities following DNA preparation by different methods. The Wilcoxon signed-rank test was used to compare within-isolate similarities to the median between-isolate similarities (Daniel, 2005). Statistical analysis was performed using Stata version 11.1 (StataCorp LP, College Station, TX).

Results

Comparison of DNA Preparation Methods. Template DNA from each SE isolate was prepared using three different methods, and RAPD-PCR was performed on duplicate samples of each isolate to quantify the within-isolate variability in banding patterns for each method. Dice coefficient similarities for duplicate runs of each isolate and extraction method are summarized in Table 1. There was no significant difference between extraction methods with respect to the within-isolate similarity values obtained using primer 1283 ($P = 0.91$), indicating that reproducibility of banding patterns was not influenced by the type of DNA template used.

In Silico Comparison of RAPD DNA Patterns for Different Typing Primers. *In silico* PCR was used to predict the number, size, and sequence of DNA amplification products that would be expected from performing RAPD-PCR on a specific SE isolate, P125109, using primers 1283, 1247, and OPA4. This analysis predicted that primers

1283 and 1247 would each produce a total of 15 DNA bands between 100 and 2,000 base pairs (bp), and primer OPA4 would produce 23 DNA bands. Based on the sequences of the predicted products, all bands expected from primers 1283 and OPA4 originated from conserved regions of the SE genome, whereas 3 of 15 (20%) DNA bands expected from primer 1247 originated from variable regions. All three variable regions were intergenic sequences having 631, 1219, and 160 nucleotides in length. Therefore, RAPD-PCR primer 1247 has a greater likelihood of detecting genetic differences between SE strains than the other two primers. The predicted DNA pattern for RAPD typing primer 1247 is shown in Figure 1, along with an actual banding pattern observed for SE isolate χ3227. The similarity of the observed patterns for duplicate PCR runs for SE isolate χ3227 (Figure 2, lanes 4 and 5) compared to the predicted RAPD DNA pattern based on *in silico* PCR (Figure 2, lane 2) was 20.0% and 38.1%, respectively.

Comparison of Within- and Between-Isolate Similarities. Primer 1247 was selected for the comparison of within- and between-isolate similarities because it had been identified as the most likely to amplify products from variable regions of the SE genome. Whole cell DNA preparation was used because it was the simplest of the three template preparation methods evaluated. Paired samples of each isolate were run side-by-side on the same gel (*Figure 2a*), and gel images were processed using Bionumerics software to mark amplicon sizes (*Figure 2b*) and calculate the within- and between-isolate similarities using dice coefficients. The similarity matrix for these isolates is shown in Table 2. The median self-similarity of isolates (76.0%) was significantly greater than the median between-isolate similarity (66.7%; P = 0.001),

however the wide variability that was observed when comparing each isolate to itself indicated that the reproducibility of RAPD-PCR was poor.

Discussion

Due to the clonal nature of *Salmonella* Enteritidis (SE), PFGE is not able to adequately discern between epidemiologically unrelated isolates. Consequently, a different approach to SE typing is needed. Phage typing is one alternative, but is not ideal because most SE isolates belong to a relatively small group of the most common phage types (e.g., PT4, PT8, and PT13). Random Amplified Polymorphic DNA (RAPD) PCR has previously been recommended as an alternative approach for typing SE isolates (Hilton et al., 1996; Hilton and Penn, 1998; Hudson et al., 2001). Several studies have reported success in using RAPD to differentiate between SE strains of different phage types (Betancor et al., 2004; Hilton et al., 1996; Hilton and Penn, 1998; Lin et al., 1996).

The primary aim for this study was to evaluate RAPD-PCR as a fingerprinting tool to identify genetic differences within SE isolates from different geographical regions or different outbreak occurrences. PCR conditions were increased in stringency in an attempt to enhance the reproducibility of RAPD banding patterns. It has been reported previously that insufficient $MgCl_2$ in the reaction mixture can result in poor amplification and that excess $MgCl_2$ increases the likelihood of non-specific amplification (Hopkins and Hilton, 2001; Lin et al., 1996). In the study described in this manuscript, a relatively low concentration of $MgCl_2$ (2 mM) was used in the master mix to increase specificity, and the number of cycles was increased to 45 to offset any resulting reduction in amplification.

The first objective of this study focused on determining whether the method of DNA template preparation had any effect on the reproducibility of the method itself. A previous study reported that the use of boiled template reduces the amount of time and labor required to perform RAPD PCR (Lin et al., 1996). In the current study, there was no significant difference between the three methods of DNA preparation with respect to the reproducibility of banding patterns. These findings are consistent with those reported by Hopkins and Hilton (2001), who found only minor variation between purified and whole cell template preparations. Likewise, Madico et al. (Madico et al., 1995) found that boiled template yielded similar results to purified template, although they noted that boiling did produce differences in some RAPD profiles, presumably due to fragmentation of template DNA. The whole cell method of DNA preparation was chosen as the preferred template type for subsequent experiments in this study based on its relatively low cost and ease of preparation.

Hudson et al. (Hudson et al., 2001) found that using a single primer was not adequate to discriminate between SE genotypes, and they reported good results using a combination of three primers: OPA4, 1283, and 1247. Others have also suggested that the use of multiple primers may be needed to identify genetic differences between strains (Tenover, Arbeit, and Goering, 1997). Even when only a single primer was used in the current study, however, the amount of within-isolate variability was unacceptably high. Using multiple primers would be expected to even further increase the within-isolate variability.

The study described in this manuscript also focused on evaluating the potential discriminatory ability of each of the three primers (1283, 1247, and OPA4) by predicting

amplification of variable regions of the genome. *In silico* PCR indicated that primers 1283 and OPA4 would amplify conserved regions of the genome suggesting that the discriminatory abilities of these primers would be minimal. Three of the predicted amplicons for primer 1247, however, targeted variable regions within the SE genome. Therefore, we elected to use primer 1247 for the comparison of within- and between-isolate similarities in this study, because it was expected to have a greater probability of identifying true genetic differences. The similarities between the actual and predicted DNA banding patterns were extremely low, however, and consequently it does not appear that *in silico* PCR provides an accurate prediction of DNA products that will be amplified by RAPD PCR.

Interestingly, Hudson, et al. 2001 reported that primer 1283 yielded more band differences in their analysis of SE isolates than did either primer 1247 or OPA4, which would not have been expected based on the results of the *in silico* PCR comparisons performed in this study. However, an evaluation of within-isolate similarities was not evaluated as part of that study, so it is not possible to determine whether the observed differences were due to genetic or method variability. Also, Hilton et al. 1998 used primer 1283 to characterize an outbreak strain of *Salmonella* Enteritidis PT4, but they concluded they were unable to differentiate that strain from other unrelated strains, and that an analysis with another independent primer may be more discriminatory (Hilton and Penn, 1998), which agrees with other past research (Louie et al., 1996).

Previous studies have reported a low genetic variability among different strains of SE using PFGE as a fingerprinting tool (Hudson et al., 2001; Millemann et al., 1995; Olsen et al., 1994; Tassios et al., 1997; Thong et al., 1995). In our analysis of within-

and between-isolate similarities, it was noted that the median within-isolate similarity of RAPD patterns was significantly higher than the median between-isolate similarity. However, the within-isolate similarities in RAPD DNA patterns were much lower than expected. In fact 9/18 (50%) isolates had a self-similarity of 75% or less, with the lowest being 61.5%. Only one isolate had a self-similarity of 100%. This observation demonstrates that DNA pattern differences generated by RAPD-PCR for SE isolates do not necessarily represent true genetic differences. Rather, many of these differences are likely to be attributable to variability in the method itself. The findings of this study are in contrast to those of Betancor et al. 2004, who reported “highly reproducible” results when using RAPD-PCR with 5 different primers, including primer OPA4, to discern differences between SE isolates. However, that study did not report a quantitative assessment of within- and between-isolate similarities.

Although previous studies have recommended RAPD analysis as an alternative genomic typing method for SE (Betancor et al., 2004; Lin et al., 1996), we found that the results of this method must be interpreted with caution. When used to identify genetic differences in SE isolates, a side-by-side comparison of the same isolates should be performed to provide an objective measure of method variation. In the absence of such a comparison, it must be recognized that any observed differences in RAPD patterns may simply be the result of method variability.

It has previously been suggested that RAPD PCR results are most reliably interpreted when all isolates are tested in a single amplification reaction and analyzed on a single agarose gel (Tenover et al., 1997). This was the same approach used in the current study, but even under these conditions a large amount of within-isolate

variability was observed. In conclusion, the reproducibility of RAPD-PCR in this study was not sufficient to reliably distinguish between related and epidemiologically unrelated SE strains.

Acknowledgement.

This work was supported by USDA NRICGP grants 2005-01378 and USDA-CREES National Integrated Food Safety Initiative Award #2007-51110-03807.

Table 2-1. *Effect of DNA Template Preparation Methods on SE RAPD Patterns.* Dice coefficient similarities (%) for duplicate samples of *S*. Enteritidis isolates (n=18) analyzed by RAPD-PCR using primer 1283 after DNA preparation by each of three methods.

Isolate	†ID	Phage Type	DNA Preparation Method		
			Purified (Commercial Kit)	Boiled	Whole Cell
A	Pt23	23	85.7	100	100
B	28b	28b	100	94.7	73.7
C	8b	8	100	80.0	90.9
D	436	4	87.0	95.2	100
E	28a	28a	100	88.9	85.7
F	SARB16	13a	95.2	90.0	95.2
G	SARB18	4	100	100	100
H	X3227	8	96.3	90.0	100
I	SE9	14b	94.7	100	90.0
J	415	4	100	100	100
K	13-1	13	100	100	94.1
L	13a-b	13a	100	94.7	88.9
M	412	4	91.7	94.7	100
N	421	4	100	100	95.2
O	413	4	100	95.2	77.8
P	417	4	88.0	90.9	100
Q	13-3	13	70.0	93.3	82.4
R	SE23	14b	94.7	87.0	95.7
		Mean	94.6	94.2	92.8
		Median	98.2	94.7	95.2
		SD	7.9	5.6	8.3

†Isolate identifiers used by Hudson et al. 2001

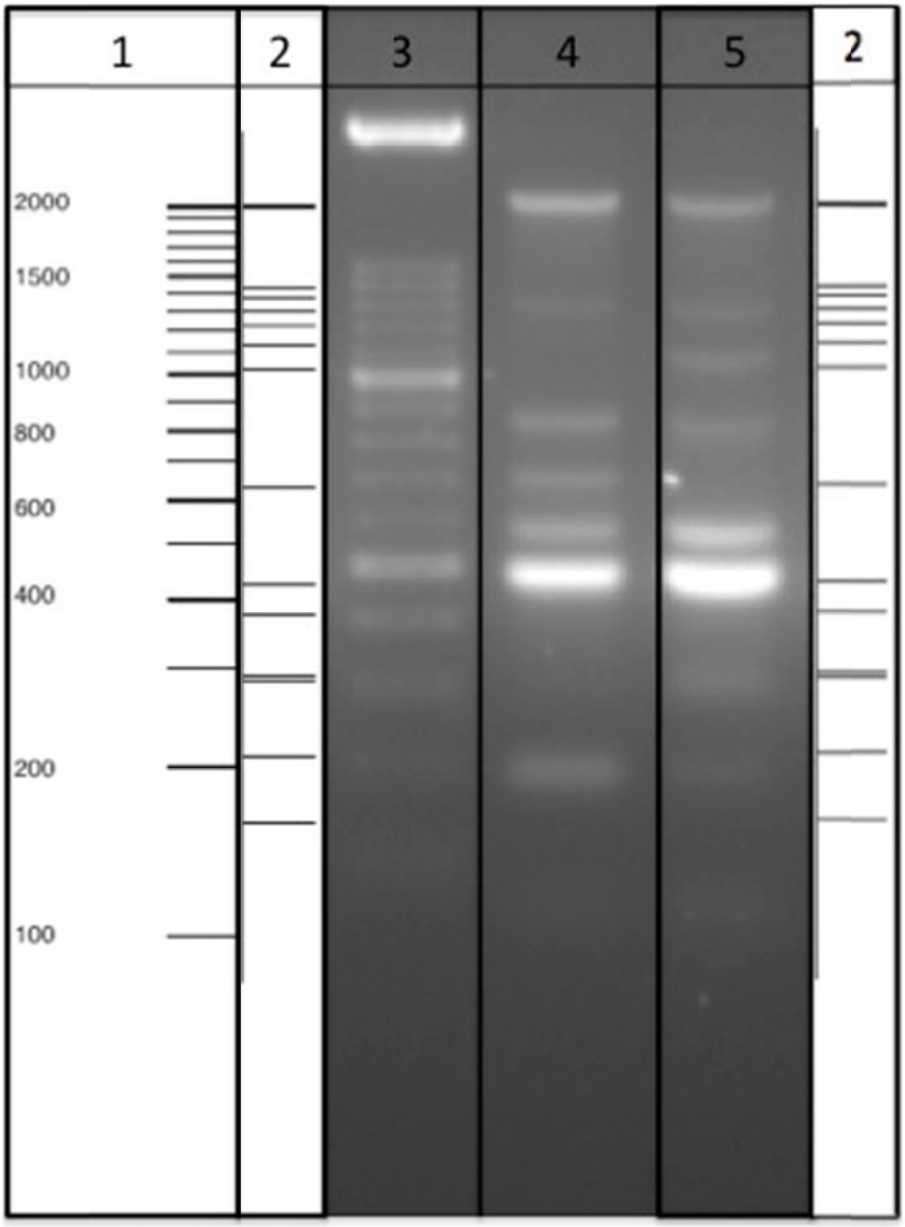

Figure 2-1. Comparison of Theoretical RAPD DNA Pattern to Actual SE RAPD DNA Pattern for SE Isolate χ3227 using Typing Primer 1247. Lane 1 is a 100 bp ladder. Lane 2 is the expected PCR product using in silico PCR. Lane 3 is DNA Molecular Weight Marker XIV (100 bp ladder, Roche Diagnostics). Lanes 4 and 5, isolate χ 3227, is the PCR product yielded from the RAPD experiment.

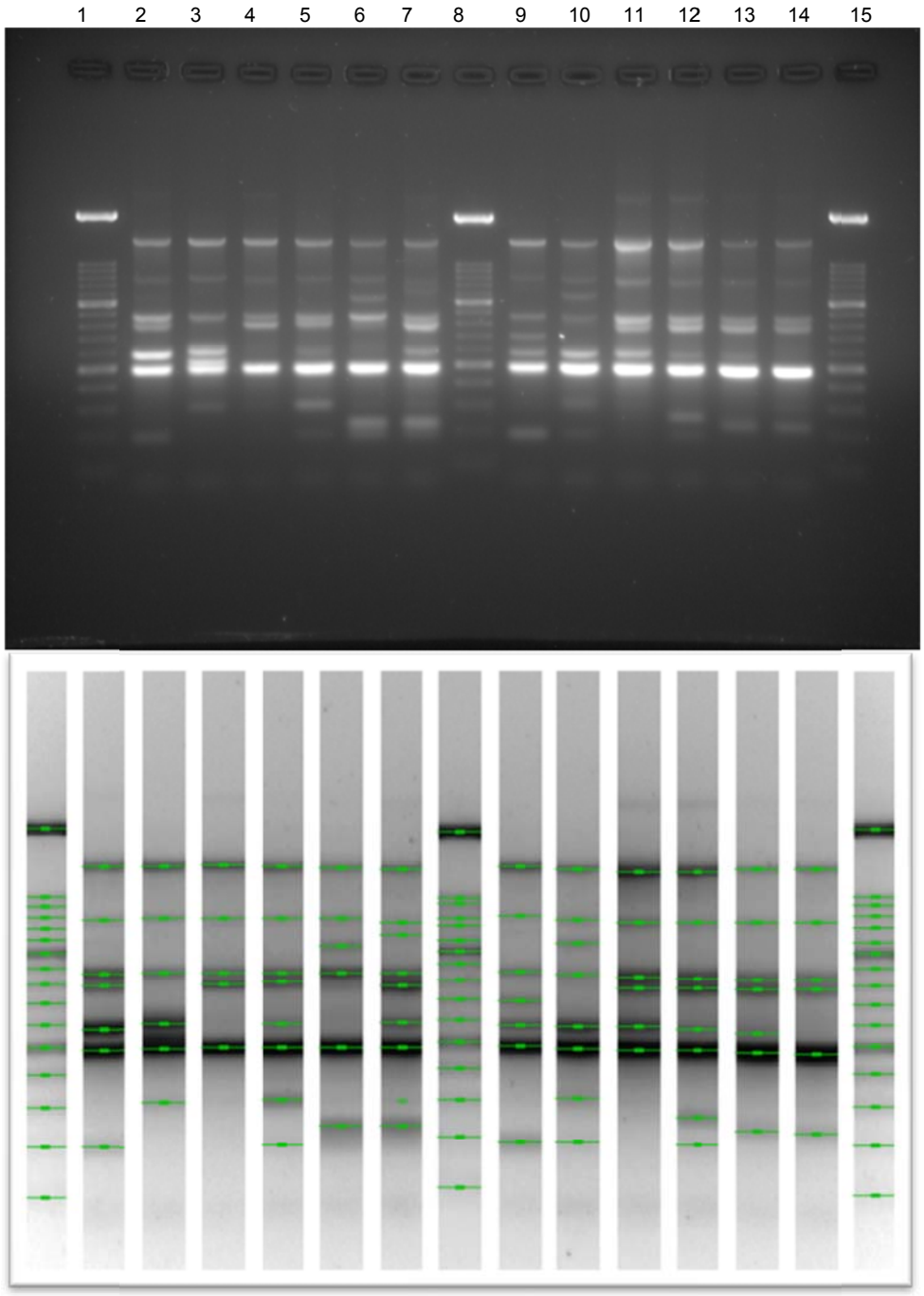

Figure 2-2a and 2-2b. Reproducibility of RAPD PCR for Typing SE Isolates. Each sample was run twice for a side-by-side analysis (2a) and processed in Bionumerics (2b). The software then compared band loci to calculate dice coefficients. Lanes 1, 8, and 15 are 100 bp ladder, lanes 2 and 3 are isolate 28a, lanes 4 and 5 are isolate 28b, lanes 6 and 7 are isolate 8b, lanes 9 and 10 are isolate χ3227, lanes 11 and 12 are isolate SARB16 and lanes 13 and 14 are isolates SARB18

Table 2-2. *Comparison of Within- and Between-SE RAPD PCR Patterns.* Dice coefficient similarities (%) comparing the RAPD DNA banding patterns for 18 *Salmonella* Enteritidis isolates typed with primer 1247. DNA template was prepared using the whole cell method. Bolded entries on the main diagonal are self-similarities for duplicate runs of the same SE isolate on the same agarose gel.

ID	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
A	**100**																	
B	90.9	**76.9**																
C	66.7	72.7	**66.7**															
D	61.5	83.3	61.5	**71.4**														
E	76.9	83.3	61.5	100	**76.9**													
F	50.0	90.9	66.7	92.3	92.3	**85.7**												
G	46.2	83.3	76.9	71.4	85.7	92.3	**92.3**											
H	61.5	66.7	61.5	85.7	85.7	76.9	71.4	**80.0**										
I	62.5	66.7	50.0	82.4	82.4	75.0	58.8	70.6	**62.5**									
J	71.4	61.5	42.9	80.0	66.7	71.4	53.3	40.0	55.6	**93.3**								
K	75.0	66.7	62.5	70.6	70.6	62.5	47.1	35.3	70.0	88.9	**77.8**							
L	42.9	46.2	42.9	53.3	53.3	57.2	53.3	26.7	33.3	87.5	77.8	**61.5**						
M	85.7	61.5	57.2	66.7	53.3	71.4	66.7	40.0	55.6	87.5	77.8	75.0	**82.4**					
N	75.0	66.7	62.5	70.6	70.6	75.0	82.4	47.1	60.0	77.8	70.0	66.7	88.9	**70.6**				
O	83.3	54.6	66.7	76.9	76.9	83.3	61.5	61.5	62.5	71.4	75.0	71.4	71.4	75.0	**62.5**			
P	61.5	66.7	46.2	57.2	57.2	76.9	57.2	42.9	47.1	80.0	70.6	80.0	80.0	70.6	76.9	**75.0**		
Q	57.2	30.8	28.6	53.3	40.0	42.9	13.3	53.3	66.7	50.0	55.6	50.0	62.5	44.5	71.4	40.0	**66.7**	
R	71.4	61.5	28.6	53.3	53.3	14.3	13.3	66.7	66.7	50.0	66.7	50.0	50.0	44.5	57.2	26.7	62.5	**75.0**

References

Altekruse, S.F., Bauer, N., Chanlongbutra, A., DeSagun, R., Naugle, A., Schlosser, W., Umholtz, R., White, P., 2006, Salmonella enteritidis in broiler chickens, United States, 2000-2005. Emerging infectious diseases 12, 1848-1852.

Anonymous, 2010, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 states, 2009. MMWR Morb Mortal Wkly Rep 59, 418-422.

Betancor, L., Schelotto, F., Martinez, A., Pereira, M., Algorta, G., Rodriguez, M.A., Vignoli, R., Chabalgoity, J.A., 2004, Random amplified polymorphic DNA and phenotyping analysis of Salmonella enterica serovar enteritidis isolates collected from humans and poultry in Uruguay from 1995 to 2002. J Clin Microbiol 42, 1155-1162.

Bikandi, J., San Millan, R., Rementeria, A., Garaizar, J., 2004, In silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. Bioinformatics 20, 798-799.

Daniel, W.W., 2005, Biostatistics : a foundation for analysis in the health sciences, 8th Edition. Wiley, Hoboken, NJ, xi, 782, 146, 784 p. pp.

Gast, R.K., 2005, Bacterial infection of eggs. Food safety control in the poultry industry (G.C.Mead, ed), 1-20.

Hilton, A.C., Banks, J.G., Penn, C.W., 1996, Random amplification of polymorphic DNA (RAPD) of Salmonella: strain differentiation and characterization of amplified sequences. J Appl Bacteriol 81, 575-584.

Hilton, A.C., Penn, C.W., 1998, Restriction enzyme analysis of randomly amplified polymorphic DNA amplicons of Salmonella enterica ser. Enteritidis PT4 and Typhimurium DT104. Lett Appl Microbiol 27, 158-162.

Hogue, A., White, P., Guard-Petter, J., Schlosser, W., Gast, R., Ebel, E., Farrar, J., Gomez, T., Madden, J., Madison, M., McNamara, A.M., Morales, R., Parham, D., Sparling, P., Sutherlin, W., Swerdlow, D., 1997, Epidemiology and control of egg-associated Salmonella enteritidis in the United States of America. Rev Sci Tech 16, 542-553.

Hopkins, K.L., Hilton, A.C., 2001, Optimization of random amplification of polymorphic DNA analysis for molecular subtyping of Escherichia coli O157. Lett Appl Microbiol 32, 126-130.

Hudson, C.R., Garcia, M., Gast, R.K., Maurer, J.J., 2001, Determination of close genetic relatedness of the major Salmonella enteritidis phage types by pulsed-field gel electrophoresis and DNA sequence analysis of several Salmonella virulence genes. Avian diseases 45, 875-886.

Kimura, A.C., Reddy, V., Marcus, R., Cieslak, P.R., Mohle-Boetani, J.C., Kassenborg, H.D., Segler, S.D., Hardnett, F.P., Barrett, T., Swerdlow, D.L., 2004, Chicken consumption is a newly identified risk factor for sporadic Salmonella enterica serotype Enteritidis infections in the United States: a case-control study in FoodNet sites. Clin Infect Dis 38 Suppl 3, S244-252.

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

Louie, M., Jayaratne, P., Luchsinger, I., Devenish, J., Yao, J., Schlech, W., Simor, A., 1996, Comparison of ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for molecular typing of Listeria monocytogenes. J Clin Microbiol 34, 15-19.

Madico, G., Akopyants, N.S., Berg, D.E., 1995, Arbitrarily primed PCR DNA fingerprinting of Escherichia coli O157:H7 strains by using templates from boiled cultures. J Clin Microbiol 33, 1534-1536.

Millemann, Y., Lesage, M.C., Chaslus-Dancla, E., Lafont, J.P., 1995, Value of plasmid profiling, ribotyping, and detection of IS200 for tracing avian isolates of Salmonella typhimurium and S. enteritidis. J Clin Microbiol 33, 173-179.

Olsen, J.E., Skov, M.N., Threlfall, E.J., Brown, D.J., 1994, Clonal lines of Salmonella enterica serotype Enteritidis documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing. J Med Microbiol 40, 15-22.

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

St Louis, M.E., Morse, D.L., Potter, M.E., DeMelfi, T.M., Guzewich, J.J., Tauxe, R.V., Blake, P.A., 1988, The emergence of grade A eggs as a major source of Salmonella enteritidis infections. New implications for the control of salmonellosis. JAMA 259, 2103-2107.

Tassios, P.T., Markogiannakis, A., Vatopoulos, A.C., Katsanikou, E., Velonakis, E.N., Kourea-Kremastinou, J., Legakis, N.J., 1997, Molecular epidemiology of antibiotic resistance of Salmonella enteritidis during a 7-year period in Greece. J Clin Microbiol 35, 1316-1321.

Tenover, F.C., Arbeit, R.D., Goering, R.V., 1997, How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. Infect Control Hosp Epidemiol 18, 426-439.

Thong, K.L., Ngeow, Y.F., Altwegg, M., Navaratnam, P., Pang, T., 1995, Molecular analysis of Salmonella enteritidis by pulsed-field gel electrophoresis and ribotyping. J Clin Microbiol 33, 1070-1074.

CHAPTER 3

CONCLUSIONS

Foodborne illness is a major public health issue in the United States. *Salmonella* is one of the leading causes of foodborne illness, causing an estimated 1.4 million illnesses and 582 deaths in the U.S. each year (Anonymous, 2007, 2008, 2009, 2010b; Mead et al., 1999). Humans can become infected with *Salmonella* from many different sources including direct contact with animals, foods of animal origin, and produce that has been contaminated with animal feces, but two of the most frequently identified sources of human illness are poultry and eggs (Anonymous, 2010c).

Salmonella Enteritidis (SE) is consistently one of the most common serotypes associated with human foodborne illness. Along with *S*. Typhimurium, it has been one of the top two serotypes involved in human infection during the past several years (Anonymous, 2007, 2008, 2009, 2010b). Since SE's emergence as a dominant serotype, it has frequently been associated with the consumption of grade A table eggs. In 2010, SE was the causative agent for a high-profile multi-state *Salmonella* outbreak that was associated with two large table egg layer operations in Iowa, which is reported to have caused over 1,800 human illnesses (Anonymous, 2010a)

In the case of an outbreak, CDC currently uses pulsed field gel electrophoresis (PFGE) to trace genetic differences between *Salmonella* isolates in determining the source. PFGE is effective in discriminating between most *Salmonella* isolates, but has

been unsatisfactory in discerning genetic differences between *S*. Enteritidis isolates due to their clonal nature (Hudson et al., 2001; Thong et al., 1995). Another tool, RAPD-PCR has been recommended as one alternative for identifying genetic differences for SE (Hilton and Penn, 1998; Hudson et al., 2001). The problem with RAPD-PCR, however, has been its low reproducibility, which prevents the results from being comparable between different trials or labs.

This study's objective was to increase the reproducibility of RAPD-PCR to make it a more reliable tool for discerning genetic differences among *S*. Enteritidis isolates. Different primers, methods of template preparation, and PCR conditions were evaluated in an effort to optimize the RAPD protocol. Even under optimized conditions, however, side-by-side comparisons of the same SE isolates yielded unacceptably low similarity values when analyzed by this method. Consequently, the ability of RAPD PCR to distinguish between epidemiologically related and unrelated SE isolates was poor.

The strength of using RAPD-PCR as a tool to analyze *S*. Enteritidis isolates lies in its short primers and the production of multiple bands for any isolate. More bands produced allows for a higher opportunity to discern genetic differences between different isolates. However, the results of the study reported here demonstrate that reproducibility of this method was unacceptably low even when working under optimized conditions. Based on this study, it is recommended that the results of RAPD-PCR testing for the discrimination of SE isolates must be interpreted with caution, and when this method is used to determine genetic differences, a side-by-side analysis of the same samples must be completed to evaluate the amount of variability due to the method itself.

References

Anonymous, 2007, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2006. MMWR Morb Mortal Wkly Rep 56, 336-339.

Anonymous, 2008, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2007. MMWR Morb Mortal Wkly Rep 57, 366-370.

Anonymous, 2009, Preliminary FoodNet Data on the incidence of infection with pathogens transmitted commonly through food--10 States, 2008. MMWR Morb Mortal Wkly Rep 58, 333-337.

Anonymous, 2010a, Investigation Update: Multistate Outbreak of Human Salmonella Enteritidis Infections Associated with Shell Eggs. Centers for Disease Control and Prevention [Online] http://www.cdc.gov/salmonella/enteritidis/.

Anonymous, 2010b, Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 states, 2009. MMWR Morb Mortal Wkly Rep 59, 418-422.

Anonymous, 2010c, Surveillance for foodborne disease outbreaks --- United States, 2007. MMWR Morb Mortal Wkly Rep 59, 973-979.

Hilton, A.C., Penn, C.W., 1998, Restriction enzyme analysis of randomly amplified polymorphic DNA amplicons of Salmonella enterica ser. Enteritidis PT4 and Typhimurium DT104. Lett Appl Microbiol 27, 158-162.

Hudson, C.R., Garcia, M., Gast, R.K., Maurer, J.J., 2001, Determination of close genetic relatedness of the major Salmonella enteritidis phage types by pulsed-field gel electrophoresis and DNA sequence analysis of several Salmonella virulence genes. Avian Dis 45, 875-886.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V., 1999, Food-related illness and death in the United States. Emerg Infect Dis 5, 607-625.

Thong, K.L., Ngeow, Y.F., Altwegg, M., Navaratnam, P., Pang, T., 1995, Molecular analysis of Salmonella enteritidis by pulsed-field gel electrophoresis and ribotyping. J Clin Microbiol 33, 1070-1074.