THE USE OF PCR-BASED METHODOLOGIES TO CHARACTERIZE *SALMONELLA* SEROTYPES OF POULTRY ORIGIN

A Dissertation

by

PHELUE NIGEL ANDERSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2008

Major Subject: Poultry Science

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ABSTRACT

The Use of PCR-Based Methodologies to Characterize *Salmonella* Serotypes of Poultry Origin. (August 2008)

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Chair of Advisory Committee: Dr. David J. Caldwell

Three studies were conducted to investigate the use of molecular techniques to identify *Salmonella* serotypes in poultry. In the first experiment, two polymerase chain reaction (PCR)-based techniques: denaturing gradient gel electrophoresis (DGGE) and polyacrylamide gel electrophoresis (PAGE) were used to analyze *Salmonella* serotype isolates from two turkey processing plants (A and B). Genotypic patterns of each isolate were compared with those of known serotypes identified by traditional antibody precipitation methods. In Plant A, four different *Salmonella* serotypes were identified: Derby, Hadar, Montevideo, and Senftenberg. In plant B, ten serotypes were identified: Agona, Anatum, Brandenburg, Derby, Hadar, Meleagridis, Montevideo, Reading, Senftenberg, and Typhimurium. *S*. Derby was predominant in Plant A (83%) while *S*. Typhimurium was the most common serotype recovered in Plant B (39%). Overall, DGGE was more sensitive than PAGE. Isolates of the same serotypes were all grouped together by DGGE, while PAGE failed to group all like serotypes.

Next, DGGE and REP-PCR were used as genotyping tools for identifying *Salmonella*. Fifty-four *Salmonella* isolates from two turkey processing plants (A and B) were evaluated. The isolates were comprised of the following serotypes: Brandenburg, Derby, Hadar, and Typhimurium (n = 6, 21, 12, and 15, respectively). Both methods were very sensitive and detected diverse fingerprint profiles among the isolates. The data suggested that REP-PCR and DGGE are useful tools for identifying *Salmonella* serotypes in research trials of this type.

The final trial was carried out to track *Salmonella* serotypes throughout an integrated poultry operation using DGGE. Four flocks were sampled from grow-out through processing. The data showed that there was correlation between *Salmonella* serotypes found on processed carcasses and during grow-out. In addition, the isolates were compared against 15 known serotypes in our data base and only *S*. Hadar from the data base matched the unknown *Salmonella* isolates.

Overall, these studies demonstrate that PCR-based methods could be considered as an alternative to conventional methods of antibody-based serotyping. Molecular methods were found to be reliable, sensitive, inexpensive, reproducible, and less labor intensive than conventional methods.

DEDICATION

This dissertation is dedicated to my loving and caring mother, Mrs. Myrtle Hunt-Henry, for all the hard work, encouragement and dedication in making me what I am today. Mom, although I am many miles away, you are always close to me at heart. As a single parent, you are always there for your children, and you taught me that I am in control of my own destiny. Today, I am proud to say I am in full control.

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TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS viii

LIST OF FIGURES x

LIST OF TABLES xi

CHAPTER

I INTRODUCTION 1

II LITERATURE REVIEW 4

Characteristics of *Salmonella* 4
Salmonella Nomenclature 5
Salmonella Grouping 8
Epidemiology of *Salmonella* 11
Clinical Characteristics of Salmonellosis 15
Route of *Salmonella* Infection 19
Treatment of Salmonellosis 22
Sources of *Salmonella* Contamination 24
Overview of the Poultry Industry 25
Salmonella Contamination of Poultry 26
Salmonella Typing Methods 35

III MOLECULAR ANALYSIS OF *SALMONELLA* SEROTYPES AT DIFFERENT STAGES OF COMMERCIAL TURKEY PROCESSING 45

Introduction 45
Materials and Methods 48
Results 52
Discussion 60

CHAPTER Page

IV EVALUATION OF REP-PCR AND DENATURING GRADIENT GEL ELECTROPHORESIS IN IDENTIFYING *SALMONELLA* SEROTYPES ISOLATED FROM PROCESSED TURKEYS 64

Introduction 64
Materials and Methods 67
Results 72
Discussion 75

V TRACKING OF *SALMONELLA* SEROTYPES FROM THE BROILER FARM TO THE PROCESSING PLANT USING PCR-BASED METHOD 80

Introduction 80
Materials and Methods 82
Results 86
Discussion 96

VI CONCLUSIONS 102

REFERENCES 105

VITA 124

LIST OF FIGURES

FIGURE Page

3-1 Dendrogram of polyacrylamide gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* serotypes recovered from two turkey processing plants (A and B) 57

3-2 Dendrogram of denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* serotypes recovered from two turkey processing plants (A and B). 59

4-1 Dendrogram shows the denaturing gradient gel electrophoresis DNA profiles (16-23S rDNA) of fifty-four *Salmonella* isolates recovered from two turkey processing plants (A and B). 73

4-2 Dendrogram shows the repetitive extragenic palindromic-PCR DNA profiles of fifty-four *Salmonella* isolates recovered from two turkey processing plants (A and B) 76

5-1 Dendrogram of flock 1 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation 87

5-2 Dendrogram of flock 2 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation 89

5-3 Dendrogram of flock 3 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation 91

5-4 Dendrogram of flock 4 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation 95

LIST OF TABLES

TABLE Page

2-1 Global prevalence (percentage) of *Salmonella* serotypes isolated from humans. 16

2-2 Global prevalence of *Salmonella* serotypes isolated from poultry origin .. 27

3-1 *Salmonella* serotypes isolated from Plant A turkey carcasses at various processing stages 53

3-2 *Salmonella* serotypes isolated from Plant B turkey carcasses at various processing stages 55

CHAPTER I

INTRODUCTION

Foodborne pathogens remain a public health threat globally. Currently, *Salmonella* is regarded as one of the primary bacterial foodborne pathogens of significance to humans (Little et al., 2007). Over the years, there has been a steady increase in the numbers of *Salmonella* cases reported. Globally, nontyphoidal *Salmonella* cases are estimated to be over 1.3 billion per year and averaging 3 million deaths (Pang et al., 1995). In the United States, there are 1.3 million cases of *Salmonella* illnesses and nearly 600 deaths occurring annually (Mead et al., 1999). The economic loss from salmonellosis is calculated at US $2.4 billion (USDA-ERS, 2005).

Several vehicles for *Salmonella* transmission to humans have been reported: pork, fish, beef, dairy products, poultry products, fruits and vegetables (Molbak, 2005). However, poultry meats and eggs are considered to be the primary hosts for salmonellosis (Capita et al., 2003; Li and Mustapha, 2002; Vadhanasin et al., 2004). Despite the recorded numbers of *Salmonella* reported in poultry, the demand for poultry products is still increasing. This increase could be attributed to the low cost of poultry meat and that consumers are more health conscious and are consuming more white-meat.

Poultry production provides a significant contribution to the agricultural sector. Broiler production worldwide was estimated to produce over 61 million metric tons for

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This dissertation follows the style of Poultry Science.

the year 2007. Of this total, the United States was calculated to produce over 16.4 million metric tons (8.88 billion birds). The US poultry industry represents an industry valued at approximately $26.8 billon, when receipts of broilers, turkeys and egg are combined (USDA-NASS, 2007).

The different stages of the commercial poultry operation provide an environment with many opportunities for *Salmonella* contamination. Generally, *Salmonella* is not considered harmful to the bird and hardly affects production (Nesbit and Ziprin, 2001). However, poultry host-specific serotypes *S*. pullorum and *S*. gallinarum cause clinical disease (Molbak, 2005). For most *Salmonella* serotypes found in poultry, it is clearly more of a public human health issue (Nesbit and Ziprin, 2001). Contamination has been found at the feed mill, breeder farm, grow-out, wild birds, farm workers, processing and retail (Bailey et al., 2001; Molbak, 2005). Similarly, *Salmonella* infected eggs in the hen's reproductive tract may contribute to progeny infection (Shivaprasad, 2000). It has been observed that *Salmonella* prevalence in the United States can be as high as 60% and 36% at grow-out and processing, respectively (Bailey et al., 2002; Molbak, 2005). Likewise, in Spain, researchers revealed a 55% and 40% incidence in carcasses and processed chicken products, respectively (Capita et al., 2003).

Since the origination of the hazard analysis critical control point (HACCP) system, poultry processing plants are continuously monitored by the government to reduce foodborne pathogens on the final products. As of 2000, the US Food Safety and Inspection Service (FSIS) imposed performance standards in federally inspected poultry plants. The allowable percentage of positive *Salmonella* on broilers and ground chicken

is 20.0% and 44.6%, respectively (Naugle et al., 2006). As such, processors and growers are forced to reduce pathogens like *Salmonella* at all stages of operation.

Historically, conventional methods of pathogen detection were the techniques of choice, but more recently, testing laboratories are utilizing PCR-based methods for this task. Phenotypic methods of testing are known to show poor reproducibility, low sensitivity, are labor intensive, expensive, and slow in pathogen identification. In contrast, genotypic methods of testing are more advantageous compared to phenotypic methods. PCR-based molecular techniques have been widely accepted as an alterative to conventional methods in pathogens detection (Jitrapakdee et al., 1995; Johnson and Clabots, 2000). Techniques such as denaturing gradient gel electrophoresis (DGGE), pulsed field gel electrophoresis (PFGE), repetitive extragenic palindromic-PCR (REP-PCR), and real-time PCR are commonly used to discriminate among bacterial species, serotypes, and strains.

The current studies will focus on the use of DGGE and REP-PCR in identifying *Salmonella* serotypes. REP-PCR targets the conserved interspersed repetitive elements that are distributed throughout the genome of prokaryotic and eukaryotic organisms (Frye and Healy, 2006; Versalovic et al., 1991). DGGE exploits the ribosomal DNA (rDNA) fragments of the conserved region that lies next to the hypervariable V3 region of the 16S rDNA gene (Muyzer et al., 1993). DGGE has been well established in gut ecology studies, but has been recently adapted for foodborne pathogens identification (Ercolini, 2004).

CHAPTER II

LITERATURE REVIEW

Characteristics of *Salmonella*

The genus *Salmonella* is phylogenically clustered in the family of *Enterobacteriaceae* (Bennasar et al., 2000; Grimont et al., 2000). *Salmonella* is characterized as ubiquitous, Gram-negative, intracellular, straight rod shaped, non-encapsulated, facultative, non-spore forming, and generally motile with peritrichous flagella (Gray and Fedorka-Cray, 2002; Kwang et al., 1996; Molbak et al., 2006; Rubin and Weinstein, 1977). The bacterium has a width of 0.7 to 1.5 µm and a length of 2.0 to 5.0 µm (Holt et al., 1994). *Salmonella* spp. are typically found in soil, water, food, and the gastro-intestinal tract of humans and other animals (Anderson and Ziprin, 2001). Most *Salmonella* are motile, with the exception of the poultry-specific serotypes of *S.* gallinarium and *S.* pullorum (Grimont et al., 2000). The organism is a facultative anaerobe that grows on food in the presence or absence of oxygen. *Salmonella* can grow within a wide range of temperatures from 8 to 45 C (Hanes, 2003), but the optimum temperature is 37 C. Typically, *Salmonella* pH growth range lies between 4.5 to 9.0 (D'Aoust, 1989); however, the most favorable pH for growth is between 6.5 to 7.5 (Garcia-Del Portillo, 1999; Ziprin, 1994). *Salmonella* is tolerant to high moisture and grows best in conditions with a water activity (a_w) of 0.93 (Gray and Fedorka-Cray, 2002; Portillo, 2000). *Salmonella* grows optimally when sodium chloride (NaCl) is between 3 to 4% and 350 mg/L of sodium nitrite ($NaNO_2$) (Portillo, 2000).

The nutrient requirement for the growth of *Salmonella* is minimal compared to other bacteria. *Salmonella* can survive on citrate (Hanes, 2003) and glucose as the only carbon and energy sources and ammonium ion for nitrogen (Grimont et al., 2000). Most *Salmonella* ferment glucose and produce hydrogen sulfide gas with or without acid; however, *S.* typhi are incapable of producing gas from fermentation of glucose (Hanes, 2003). Furthermore, most *Salmonella* are unable to catabolize lactose and sucrose. *Salmonella* are non-tolerant to oxidase and can convert nitrate to nitrite (Hanes, 2003). In addition, lysine and ornithine are decarboxylated by *Salmonella*. *Salmonella* do not hydrolyze urea, nor do they deaminate tryptophan or phenylalanine (Anderson and Ziprin, 2001; Grimont et al., 2000). These unique characteristics provide a clear form of demarcation when identifying *Salmonella* from other closely related organisms. The type of media that are used for growth may influence *Salmonella* colonies appearances. For example, growing *Salmonella* on xylose lysine terigitol-4 (XLT-4) will display black-centered colonies (Grimont et al., 2000), whereas, on brilliant green agar (BGA) the colonies appear to be pink.

Salmonella **Nomenclature**

Preliminary *Salmonella* research is dated back to 1880, when the bacteria were isolated from a person who died from typhoid fever. Subsequently, in 1886, Daniel E. Salmon and colleagues isolated from swine the organism currently known as *Salmonella* choleraesuis, which was in 1880 believed to be the causative agent for hog cholera (Grimont et al., 2000; Le Minor, 1991).

Salmonella nomenclature is credited to early pioneers such as White, Borman, Kauffmann, Edwards, and Le Minor (Grimont et al., 2000). Over the years, the *Salmonella* nomenclature system has been revised several times (Euzeby, 1999). Started by White and followed by Kauffmann, a one serotype-one species concept, known as the Kauffmann and White system, was created based on the somatic (O), flagella (H) and surface envelope (Vi) antigens (Brenner et al., 2000; Grimont et al., 2000). Not long after its creation, the one serotype-one species concept was discontinued, since most serotypes were closely related (Andrews and Baumler, 2005). Subsequently, a two species system was proposed to classify *Salmonella* (Andrews and Baumler, 2005; Grimont et al., 2000; Reeves et al., 1989). Currently, if the one-serotype-one species were still endorsed by bacteriologist, over 2541 different serotypes would be documented (Brenner et al., 2000; Grimont et al., 2000; Popoff et al., 2004). The Kauffmann-White system has been endorsed by the Centers for Disease Control (CDC) since 2003 (CDC, 2004).

S. choleraesuis was the species name proposed to correct the deficiency in the nomenclature. However, there was still confusion with this proposal since *S. choleraesuis* was also a serovar (Grimont et al., 2000). Subsequently, *S. enterica* was proposed as a definitive species to alleviate confusion and help define this system of nomenclature for broad acceptance. Correspondingly, the genus *Salmonella* has been divided into two species: *S. enterica* and *S. bongori*. Currently, *Salmonella enterica* consists of six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica* (Grimont et al., 2000; Popoff et al., 2004; Reeves et al., 1989). *S. bongori* was

previously thought to be a subspecies of S. *enterica*. However, with DNA-DNA hybridization, it has been demonstrated that *S. bongori* is distantly related to the other six subspecies (Reeves et al., 1989). *S. bongori* and the subspecies of *S. enterica* can be written using either names or Roman numerals or both. For example, *S. enterica* subsp. *enterica* **I**, *S. enterica* subsp. *salamae* **II**, *S. enterica* subsp. *arizonae* **IIIa**, *S. enterica* subsp. *diarizonae* **IIIb**, *S. enterica* subsp. *houtenae* **IV**, *S. enterica* subsp. *bongori* **V,** and *S. indica* **VI** (Brenner et al., 2000).

Prior to 1968, the Kauffmann-White system identified serotypes in subspecies (I-VI) and species (V) by names. Subsequently, only serotypes from *S. enterica* subsp. *enterica* (I) were referred to by names, and all other serotypes from subspecies (II, IIIa, IIIb, IV and VI) and *S.* bongori (V) were described by antigenic formulas (Brenner et al., 2000). To date, 2541 different *Salmonella* serotypes are reported to affect humans and other animals. A recent report has shown the number of serotypes found in each subspecies and species and has been documented as follows: subspecies *enterica* (1504), *salmanae* (502), *arizonae* (95), *diarizonae* (333), *houtenae* (72), *indica* (13), and species *S. bongori* (22) (Popoff et al., 2004). Globally, it is estimated that approximately 99% of all human salmonellosis is attributed to *S. enterica* subsp. *enterica* (Aleksic et al., 1996; CDC, 2004).

Annually, new *Salmonella* serotypes are identified following their isolation from humans, other animals, or the environment. *Salmonella* serotypes in the past were named with reference to the disease caused or animal species from which the bacterium was first isolated. In human medicine, serotype names were linked to the bacteria that caused

the infection, such as *S.* paratyphi A and *S.* typhi. Similarly, the veterinary community used serotype names that were more related to the animal species from which the bacteria were isolated: For example, *S.* bovis, and *S.* gallinarum. Currently, some new serotypes are named with reference to the geographical location where the pathogen was isolated, thus serotypes such as *S.* dublin, *S.* panama, *S.* paul and *S.* heidelberg (Anderson and Ziprin, 2001; Ziprin, 1994).

There have been additional modifications in the way the serotype names are written or reported. Serotype names are no longer written using italics. Only the genus is italicized and the first letter of the serotype is capitalized. For example, *Salmonella* enterica subsp. *enterica* serotype *typhimurium* is now written as *Salmonella enterica* subsp. *enterica* Typhimurium or for simplicity *S.* Typhimurium (Andrews and Baumler, 2005; Brenner et al., 2000). From this point onward in this manuscript, the new nomenclature will be adopted with regard to serotype names.

Salmonella Grouping

The Kauffmann-White scheme was established on the principle of antigen-antibody interaction and the chemical composition of surface antigens determines the specificity of the interaction (Guthrie, 1992). *Salmonella* has two surface antigens, somatic (O) and flagella (H), and may have a surface envelope (Vi), which are used to divide the bacteria into serogroups.

The somatic antigens contain lipopolysaccharide (LPS) that are present on the cell membrane of the bacteria. Furthermore, O-antigens are very heat stable and alcohol resistant. The somatic component LPS is subdivided into three segments: lipid A, R-

core, and O-polysaccharide. Lipid A connects the other two segments to the endotoxin complex and is composed of fatty acid moieties. The R-core is the middle section of the LPS and functions to bridge the gap between Lipid A and the O-polysaccharide. The other shell (O-polysaccharide) controls the specificity of the O-antigen and is composed of repeating sugar molecules (Rubin and Weinstein, 1977), glucose, abequose, galactose, rhamnose, mannose, and tyvelose, that determine the antigen subgroup (Andrews and Baumler, 2005; Grimont et al., 2000).

The O-antigen is made up of thirteen serogroups or factors (A, B, C1, C2, D, E1, E2, E3, F, G, H, I, and others) and is classified based on antisera-antigen agglutination (Guthrie, 1992; Rubin and Weinstein, 1977). Most serotypes of *Salmonella* belong to the first eight factors (Chiu et al., 2004; Guthrie, 1992). Each alphabetical serogroup contains several antigen groups that are designated by Arabic numbers (1 to 67) and are numbered according to the time of discovery (Anderson and Ziprin, 2001; Edward and Ewing, 1972a). *Salmonella* serotypes differ from each other by an immuno-dominant antigen that binds with high affinity to an antibody. For example, serogroups A, B, C1, C2, and D are identified by immuno-dominant antigens 2, 4, 7, 8, and 9, respectively (Andrews and Baumler, 2005; CDC, 2004; Guthrie, 1992; Rubin and Weinstein, 1977).

H-antigens are found on the flagella of the bacterial cell and are heat and alcohol labile. This antigen is composed of the protein flagellin, which is primarily isolated from the flagella of the bacterium. The H-antigen contains two flagellins known as H1 and H2 (phase 1 and 2) positioned at a distance from each other on the chromosome (Andrews and Baumler, 2005; CDC, 2004). In the *Salmonella* antigenic formula, H1 is written in

lower case letters, whereas the H2 gene is designated by Arabic numerals (Andrews and Baumler, 2005; Edward and Ewing, 1972a). Serovars that contain only one of the flagellin antigens are monophasic and when both flagellin antigens are present, such a serovar is referred to as diphasic. The predominant monophasic serotypes are *S.* Enteritidis, *S.* Typhi, and most subspecies of *S. enterica* subsp. *arizonae* and *S. enterica* subsp. *houtenae* (CDC, 2004).

The structure of the Vi-antigen is composed of a linear homopolymer of 2–aceteamido-2-deoxy-D-galacturonic acid linked by α(1-4) bonds (Grimont et al., 2000). Heat alters the ability of *Salmonella* to agglutinate the Vi-antiserum and causing it to bind instead to O-antiserum. However, when treated with alcohol prior to boiling, the antigen remains unchanged (Edward and Ewing, 1972b). The Vi-surface antigen is associated with virulence genes found in some *Salmonella* serotypes. Mice that possess the Vi-antigen were shown to be more virulent when compared to the control (Edward and Ewing, 1972b). The Vi-antigen is more prevalent in serotypes *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi C, and *S.* Dublin (Grimont et al., 2000; Guthrie, 1992).

Taking these characteristics into consideration, each *Salmonella* serotype is represented by a unique antigenic formula. The O-antigen is written first, next, the H1 gene antigen, and finally, the H2 gene antigen. The O-antigen is separated from the H1 antigen by a colon, and the H2 antigen is separated from the H1 antigen by a colon (Brenner et al., 2000; Reeves et al., 1989). For example, the antigenic formula for *S.* Typhimurium is written as (1,4,5,12: i:1,2). The O-antigen segment contains the factors (1,4,5,12), the H1 antigen consists of factor (i), and the H2 antigen (1,2). Furthermore,

not all *Salmonella* serotypes contain the O-antigen and serotypes lacking the O-antigen are termed as variant (Var.) serotypes. For example, when *S.* Typhimurium is missing the O5-antigen, the serotype is referred to as *S.* Typhimurium Var. Copenhagen (Andrews and Baumler, 2005).

Epidemiology of *Salmonella*

Salmonella is one of the leading causes of foodborne illnesses worldwide. Salmonellosis, the disease caused by *Salmonella*, is linked to humans consuming contaminated food or drinking water and to a lesser extent people mingling with some animals. It has been well documented that the common sources of salmonellosis are poultry meat and eggs (Chang, 2000; Guard-Petter, 2001; Kimura et al., 2004), beef (Wong et al., 2007), milk and cheese (CDC, 2007a), pork (Ojha and Kostrzynska, 2007), and fish (Greenlees et al., 1998). In addition, fruits and vegetables are also implicated as vehicles for *Salmonella* transmission, for example, lettuce and spinach (Guentzel et al., 2008), cantaloupe (Ukuku, 2006), and fruits (Heaton and Jones, 2007). Furthermore, pets such as turtles have been reported to cause salmonellosis in humans, especially children. Consequently, some states have banned the sale of small turtles as pets (CDC, 2007b). Unfortunately, poultry meat and eggs have gained considerable attention over the years and have been implicated as the major pubic health hazard to food safety (Li and Mustapha, 2002; Vadhanasin et al., 2004).

Salmonella is ecologically divided into three categories based on host adaptation and preferences. The first group of *Salmonella* within this categorical subdivision is "highly adaptive to man." This group contains the serotypes *S.* Typhi, *S.* Paratyphi A, *S.*

Paratyphi B, and *S.* Paratyphi C. These serotypes are the causative agents of typhoid fever. The second group consists of *Salmonella* "highly adaptive to specific non-human hosts" (intermediate). Within this group are serotypes associated with a specific animal species, including *S.* Abortusequi in sheep, *S.* Dublin in cattle, *S.* Abortusovis in swine, and *S.* Pullorum or *S*. Gallinarium in poultry. It is rare for these serotypes to cause human illness. The third group includes serotypes "unadapted to specific host." Most of these ubiquitous serotypes fall within the serotypes unadapted to specific host group and are responsible for dramatic salmonellosis (Rubin and Weinstein, 1977; Wallis, 2006; Ziprin and Hume, 2001).

A *Salmonella* surveillance system is an integral component of epidemiology to adequately study, document, and control the pathogens. Several countries have either a national or a regional *Salmonella* data bank to which outbreaks are reported. European countries have well established data bases of human salmonellosis cases (Schlundt et al., 2004). Similarly, the United States, since 1962, has implemented a *Salmonella* surveillance system that tracks and monitors *Salmonella* outbreaks, implements control measures, and identifies the serotypes involved (Olsen et al., 2001). However, in less industrialized nations, such as in Africa, there are limited *Salmonella* data available (Crump et al., 2004). These countries lack funding, proper testing laboratories, and personnel to adequately isolate and characterize the organism.

S. Typhi, the causative agent of typhoid fever was the most predominant *Salmonella* serotype recovered from the 1880's to the 1950's (Tauxe, 1991). Typhoid fever is prevalent in countries with poor sanitation, over crowding, and contaminated

food or water. In industrialized countries, typhoid fever is linked to persons traveling abroad (Ziprin and Hume, 2001). During the 1920's, *S.* Typhi incidence per 100,000 residents was over 40 cases, whereas, in 1955, incidence decreased to 1 case. By 1966, *S.* Typhi was essentially eradicated in the United States (Molbak et al., 2006). The low incidence of *S.* Typhi detection could be associated with improvements in waste disposal, clean water, food handling, personal hygiene, and patient care (Guthrie, 1992; Molbak et al., 2006).

It is very difficult to get a true estimate of foodborne illness since only severe cases are ever reported. To obtain an estimate for *Salmonella,* it is assumed that for each case of *Salmonella* confirmed by a laboratory test, there are 38 other unconfirmed cases (Voetsch et al., 2004). According to the World Health Organization (WHO), the global estimate for typhoid fever is 16.6 million and averaging 600,000 deaths. In a more recent study, the cases of typhoid fever are estimated to be 21.6 million (Crump et al., 2004). The author suggested that the difference of the 5 million cases between the studies could have been that there was a 20% increase in global population over the period. Also, for every case of typhoid fever it is assumed that there is 0.25 cases of paratyphoid fever occurring (Crump et al., 2004). In contrast, nontyphoidal *Salmonella* is estimated to be responsible for 1.3 billion cases, and approximately 3 million deaths annually (Pang et al., 1995). A global epidemiology study of *Salmonella* by Crump and colleagues (2004) divided the world into six regions. The incidence levels per 100,000 persons for each region were as follows: North America, Europe, Oceania, Africa, Latin America

including the Caribbean, and Asia with 0.15, 2.6, 15.4, 49.8, 53.1, and 274.3, respectively (Crump et al., 2004).

In the United States, from 1996 to 1999, foodborne illnesses from *S.* Typhi were estimated to be fewer than 700 cases, with 492 persons were hospitalized resulting in 3 deaths. In contrast, nontyphoidal *Salmonella* in the United States was estimated to cause 1.34 million cases, of which 16,430 persons were hospitalized and 553 deaths reported (Mead et al., 1999). Annually, salmonellosis costs the United States economy $2.4 billion, resulting from medical cost, loss of productivity, and premature death (USDA-ERS, 2005). In Denmark, from 1991 to 2000, there were approximately 28,000 cases of nontyphoidal *Salmonella* and 5000 patients hospitalized (Helms et al., 2006).

A worldwide survey from the period 2000 to 2002 of forty-nine countries reported *Salmonella* serotypes (human and non-human) to the WHO global *Salmonella*-survey data bank. The data had the highest *Salmonella* serotyped recorded in 2000 compared to other years for both humans and non-humans. On average, the five top *Salmonella* serotypes primarily isolated from humans were Enteritidis, Typhimurium, Newport, Heidelberg, and Infantis (Galanis et al., 2006; Herikstad et al., 2002). Interestingly, only in North America was *S.* Enteritidis not identified as the primary human serotype. *S.* Typhi is very prevalent in Asia, Caribbean, South America, and Africa, however, no mention was made of this serotype for the survey. The author suggested that no *S*. Typhi was serotyped and only *Salmonella* isolates that were serotyped were included in the data. The top non-human isolates were Typhimurium, Heidelberg, Enteritidis, Infantis, and Newport. Interestingly, the same serotypes from

human and nonhuman sources were reported, but in different order. A comprehensive list of the humans serotypes isolated are reported in Table 2-1 (Galanis et al., 2006). Some *Salmonella* serotypes are restricted to particular regions, for example, *S.* Weltevreden is more common in S.E. Asia and *S.* Marina is primarily found in marine iguanas of South America and is rarely detected in other regions (Galanis et al., 2006).

Clinical Characteristics of Salmonellosis

Clinical *Salmonella* infection is commonly divided into four disease syndromes: gastroenteritis, bacteremia (with or without focal extraintestinal infection), enteric fever, and an asymptomatic carrier state (Gray and Fedorka-Cray, 2002; Guthrie, 1992; Rubin and Weinstein, 1977). Other researchers have divided *Salmonella* illness into two broad categories: nontyphoidal salmonellosis (gastroenteritis) and typhoidal (enteric fever) (Garcia-Del Portillo, 1999; Ziprin and Hume, 2001). According to the literature of Gray and Fedorka-Cray (2002), the authors suggested that some *Salmonella* serotypes are linked to specific clinical syndromes. For example, *S.* Typhimurium, *S.* Enteritidis, and *S.* Newport are primarily responsible for human and non-human gastroenteritis. Similarly, *S.* Typhi and Paratyphi serotypes are associated with human enteric fever, whereas *S.* Choleraesuis is associated bacteremia in pigs.

Gastroenteritis was symptomatic of approximately 15% of foodborne illness in the United States. The incubation time for the onset of the illness is from 6 to 48 hours after the ingestion of contaminated food or water (Gray and Fedorka-Cray, 2002; Molbak et al., 2006; Rubin and Weinstein, 1977; Ziprin and Hume, 2001). The short incubation time for salmonellosis to occur could be influenced by the dosage of

Table 2-1. Global prevalence (percentage) of *Salmonella* serotypes isolated from humans. [Adapted from Galanis et al., (2006)].

Salmonella Serotype	Regions				
	Africa	Asia	Europe	Latin America & Caribbean	N. America
Anatum	0	6	0	0	0
Enteritidis	26	38	85	31	21
Hadar	0	0	2	0	0
Heidelberg	0	0	0	0	10
Infantis	4	0	2	0	0
Javiana	0	0	0	0	4
Montevideo	0	0	0	6	0
Newport	0	0	0	0	15
Paratyphi B	0	0	0	5	0
Rissen	0	6	0	0	0
Typhi	8	0	0	13	0
Typhimurium	25	6	5	18	29
Virchow	0	0	2	0	0
Weltevreden	0	6	0	0	0
Others	37	38	4	27	21
Total Percentage	100	100	100	100	100

Salmonella ingested (Molbak et al., 2006) and the health status of the host (Ziprin and Hume, 2001).In most cases, the first symptom of salmonellosis exhibited is diarrhea and is usually resolved within a week. Subsequently, the patient will display symptoms of abdominal cramps, nausea, vomiting, headaches, fever, chills, myalgia, and pain in joints (Molbak et al., 2006; Ziprin and Hume, 2001). Occasionally the patient feces will contain blood and is a good indicator for laboratory analysis of the stool (Molbak et al., 2006). Mortality among patients with gastroenteritis syndrome is minimal and is distinctively seen in patients infected with very pathogenic serotypes of *Salmonella* (Ziprin and Hume, 2001).

Salmonella bacteremia syndrome is characterized by the presence of the bacteria in the blood or circulatory system following gastroenteritis and the resulting syndrome can last for weeks (Rubin and Weinstein, 1977). *S.* Choleraesuis is more commonly observed in swine, nevertheless, the organism is very virulent in humans causing *Salmonella* bacteremia. A survey of *Salmonella* bacteremia in Taiwan hospitals, from 1994 to 2004, showed consistent increases in all years with the exception of 1998 (Jean et al., 2006). The symptoms of bacteremia include fever, diarrhea, joint pains, abdominal pain, nausea, and vomiting. As expected, diarrhea is three times higher in children compared to adults (Chiu et al., 2006). Mortality from bacteremia is more than twice the incidence of typhoid fever and occasionally up to six times greater than other syndromes (Jean et al., 2006; Rubin and Weinstein, 1977). It is interesting to note that *Salmonella* bacteremia contributes to numerous focal infections: osteomyelitis, inflamed

pancreas, abscesses of skin, tumors, and many other secondary complications (Rubin and Weinstein, 1977).

Enteric fever causes systemic infection and displays symptoms such as fever and abdominal disturbances. Enteric fever associated with *S.* Typhi is known as typhoid fever. In enteric fever, the bacteria migrate from the gastrointestinal tract to the lymphatic system, blood, spleen, and liver resulting in systemic infection (Ziprin and Hume, 2001). The incubation time for the onset of typhoid fever is estimated to be between three days and a month. Whereas, the incubation period for paratyphoid fever is from one to ten days (Molbak et al., 2006). Following incubation, symptoms such as headache, bradycardia, constipation, diarrhea, muscle soreness, malaise, rose spots, chills, and fever will be exhibited (Molbak et al., 2006; Ziprin and Hume, 2001). Patients with typhoid fever may have a fever of 103-104 F at the first one- to two-weeks phase and can gradually increase over time.

Asymptomatic carriers are persons capable of transmitting the illness to others without displaying any symptoms of the illness. According to Rubin and Weinstein (1977), the carrier is exposed to limited CFU of *Salmonella* initially that are too low to initiate disease. In nontyphoidal patients, an asymptomatic state can develop in about four to five weeks following gastroenteritis. To be describe as a chronic carrier, the patient should be colonized with *Salmonella* in the feces or urine for over one year (Molbak et al., 2006; Rubin and Weinstein, 1977). Chronic asymptomatic carriers are of extreme concern for public health officials, particularly if they are employed as food handlers and health workers (Ziprin and Hume, 2001). Without proper personal hygiene,

there is a high probability that the carrier can infect others with the bacteria. The case involving an asymptomatic carrier commonly referred to as "Typhoid Mary" in New York is a perfect example (Guthrie, 1992; Molbak et al., 2006). The Irish descendant worked as a cook in New York in the early 1900's, she appears to be healthy, but continues to infect others with the *S*. Typhi bacteria. Approximately 1 to 4% of *S.* Typhi patients develop into a chronic asymptomatic state, however, a lower percentage was observed in *S.* Paratyphi patients. Interestingly, in such individuals *Salmonella* is commonly localized in the gall bladder and develops resistance to the bile and the alkaline environment. It subsequently proliferates and evades host defense mechanisms without being destroyed (Guthrie, 1992).

Route of *Salmonella* Infection

The typical route for *Salmonella* to enter the host is orally and is initiated by consuming contaminated food or water (Darwin and Miller, 1999; Mastroeni, 2006; Ohl and Miller, 2001). The severity of the outbreak is dependent on the health status of the host. For example, babies, young children, elderly, and immunocompromised patients are more susceptible to salmonellosis (D'Aoust and Maurer, 2007). Infective dose in an outbreak can vary widely and has been reported to range from 10^1 to 10^{11} cells, often depending on the food item serving as the vehicle for infection (D'Aoust and Maurer, 2007). The infective dose has been shown to be lowered by consuming liquid food and anti-acid products (Darwin and Miller, 1999). Under normal circumstances, a large number of CFU may be required to combat the acidic environment of the gastric region and hostility from the natural microflora in the intestine and cause infection (Darwin and

Miller, 1999). From the mouth following ingestion, *Salmonella* travels via the esophagus to the stomach, which is very acidic (pH 1.5-2.0). Generally, the organism is poorly adapted to pH lower than 4.0, however, *Salmonella* can develop an acid tolerance response to provide protection from the acid stress environment (Hu and Kopecko, 2003).

By peristalsis, *Salmonella* are transported from the stomach to the small intestine. Interestingly, in mice, only about 5% of the bacteria that survived the acidic environment ever reach the small intestine and the gut associated lymphoid tissues (GALT) (Baumler et al., 2000). Intestinal epithelial cells provide a physical barrier against evading bacteria. Furthermore, the host's innate defense system through paneth cells discharge large amounts of antimicrobial peptides to destroy the pathogen (Cash and Hooper, 2005). Once *Salmonella* overcome host defense mechanisms, it will move to the Peyer's patches within the intestinal mucosa of the ileum. Peyer's patches are the primary sites of *Salmonella* invasion through the intestinal epithelium (Baumler et al., 2000). Peyer's patches contain specialized microfold (M) cells that are located in the follicle-associated epithelium (FAE) (Hanes, 2003; Hu and Kopecko, 2003; Ohl and Miller, 2001). These M cells contain large quantities of glycoconjugate on the apical surface (Baumler et al., 2000). The function of the M cells is to sample the luminal content of the epithelium and return the antigens to the FAE cells (Darwin and Miller, 1999; Garcia-Del Portillo, 1999). *Salmonella* have been shown to colonize the M cells within 30 min post infection (Hanes, 2003).

Following *Salmonella* invasion of the intestinal epithelium, the bacteria are attached to the mucous layer surface by the numerous fimbriae present on the outer membrane of the cell. According to Darwin and Miller (1999), there are four types of fimbriae: type 1(Fim), plasmid-encoded (PE), long polar (LP), and thin aggregative (curly) fimbriae. Subsequent to *Salmonella* attachment, the bacteria destroy the brushborder of the intestinal epithelium, thus altering the cytoskeletal rearrangement of the actin filament, an event known as membrane ruffling (Garcia-Del Portillo, 1999; Gray and Fedorka-Cray, 2002).

The following paragraphs describe the integrated host response of the immune system to the invasive *Salmonella*. An inflammatory response can then be activated upon the interaction of *Salmonella* and the epithelial cell. The interaction facilitates the recruitment of polymorphonuclear (PMN) lymphocytes to the inflamed region (Garcia-Del Portillo, 1999; Gray and Fedorka-Cray, 2002). Antigen presenting cells (APC) present the antigen (microbe) to phagocytes. The APC (B cells, T cells, macrophages and dendritic cells) are located below the FAE of the Peyer's patches (Gray and Fedorka-Cray, 2002). APC are responsible for the activation on T and B cells of the adaptive immune system (Cash and Hooper, 2005).

Numerous cytokines are released in response by the host in defense against the pathogen. The T-cell is major histocompatibility complex (MHC) restricted and binds only to specific T-cells. MHC-II binds to the CD4 T-cell and MHC-1 binds to the CD8 T-cell. T-cell activation involves the APC binding to the naive T-cell (CD4 and CD8). CD4 T-cells then differentiate into effector cells that activate macrophage and B-cells,

while CD8 T-cells differentiate into CD8 effector cells and function to kill infected target cells and activate macrophages. Memory cells (CD 4 and CD 8) boost the immune system the next time they encounter the pathogen (Abbas and Lichtman, 2003). The release of cytokines such as interleukin-2 (IL-2) stimulates clonal expansion and differentiation of the T-cells. Similarly, IL-8 functions to send PMN to the region of pathogen and epithelial cell contact (Gray and Fedorka-Cray, 2002).

Systemic infection develops if the host is unable to prevent the microbe from multiplying in the Peyer's patches and subsequently invading the host (Baumler et al., 2000). Systemically, *Salmonella* is then transported from the intestine to the vena cava via the mesenteric lymph nodes (Baumler et al., 2000; Mastroeni, 2006). In the lymphatics, professional killing cells guide the *Salmonella* to a low nutrient and antimicrobial environment. Thus, exposing the microbe to nitrogen, oxygen and non-oxygen reactive mechanisms will facilitate engulfing of the pathogen (Hanes, 2003; Ohl and Miller, 2001). Interestingly, *Salmonella* is more tolerant to non-oxygen than oxygen reactive mechanisms (Hanes, 2003). *Salmonella* not killed by the host defense system has to be removed from the blood and reside in the liver, spleen, bone marrow and ceca of birds (Mastroeni, 2006). In the liver and spleen, *Salmonella* will survive and replicate in macrophage, PMN, and dendritic cells (Baumler et al., 2000; Gray and Fedorka-Cray, 2002; Mastroeni, 2006).

Treatment of Salmonellosis

To adequately treat salmonellosis, consideration should be given to the symptoms and syndrome exhibited by the patients. Illness from gastroenteritis is

commonly treated by using “supportive therapy”, which includes the administration of electrolytes and fluids to reverse dehydration. It not necessary to provide antimicrobial therapy, since the illness is self limiting, furthermore, antibiotics will reduce *Salmonella* shedding and increase recovery time (Gray and Fedorka-Cray, 2002; Hanes, 2003). Systemic infection, focal infection, and bacteremia are best treated using antimicrobial agents (Hu and Kopecko, 2003; Ziprin, 1994; Ziprin and Hume, 2001). To treat systemic infection, the drugs should penetrate the phagocytic cells and destroy the bacteria at the point of replication. Drugs such as quinolones and ciprofloxacin have gained favorable response in patient therapy (Ziprin and Hume, 2001). A chronic asymptomatic carrier is best treated by undergoing cholecystectomy, however, antimicrobial therapy using ampicillin, amoxicillin, and fluoroquinolones has provided limited success (Hu and Kopecko, 2003). A focal abscess is best treated by surgically draining the inflamed organ (Gray and Fedorka-Cray, 2002).

Currently, there is a global awareness of the overuse of antibiotics both in humans and in domestic animals and there is a high probability for the development of antimicrobial resistance in bacteria from such overuse. Animal agriculture is targeted as the major culprit in the development of antibiotic resistance in humans. Presently, there are fewer antibiotics available to treat salmonellosis compared to a decade ago, as a result of the development of resistance to several of these once highly effective drugs. There is an abundance of literature available focusing on antibiotic resistance of *Salmonella* spp. (Dechet et al., 2006; Esaki et al., 2004; Rabatsky-Ehr et al., 2004; Threlfall et al., 1996). *S.* Typhimurium definitive type 104 (DT104) is exhaustively

documented in the literature. *S.* Typhimurium DT104 has been reported to have numerous chromosomal resistance genes (Gray and Fedorka-Cray, 2002). In England and Wales, *S.* Typhimurium DT104 has shown increased multiple drug resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (Threlfall et al., 1996). Thus, treating patients with *Salmonella* resistant strains is more complex and antibiotic sensitivity tests are needed prior to drug therapy. To reduce the burden of increasing antibiotic resistance of *Salmonella* spp. in humans, it may be useful to focus on antibiotic use in agriculture. The problem could be curtailed by only administering antibiotics to animals for clinical cases not for growth enhancement (Dechet et al., 2006).

Sources of *Salmonella* Contamination

Salmonella has been frequently reported in products of animal and plant origin. Studies have shown that dairy products (Donnelly, 1990), beef (Small et al., 2006), fish (Heinitz et al., 2000), pork (Wong et al., 2007), poultry meat and eggs (Ghafir et al., 2005), and fruits and vegetables (Heaton and Jones, 2007) are all vehicles for *Salmonella* transmission to humans. Of these, poultry meat and eggs are frequently considered to be the major vehicles for human infection (Capita et al., 2003; Li and Mustapha, 2002; Vadhanasin et al., 2004). There is an extensive data base of literature available that examines the impact of *Salmonella* on the poultry industry. Generally, *Salmonella* is not considered harmful to the bird and it hardly affects production. It is clearly more of a public human health issue (Nesbit and Ziprin, 2001).

Overview of the Poultry Industry

Poultry production is widely practiced around the world, ranging from subsistence farming to large commercial enterprises generating billions of dollars worldwide. In 2007, an estimate by the United States Department of Agriculture-Foreign Agricultural Service (USDA-FAS) reported that poultry meat was the second most consumed meat worldwide. The meat consumption data was expressed as per 1000 metric tons and consumption was as follows: pork, 98,136 (47%); broiler, 51,725 (28%); and beef combined with veal, 51,725 (25%). Broiler meat consumption per capita for most countries showed a gradual increase from year 2002 to year 2006. The five top countries where broiler meats were consumed (kilograms per person) are The United Arab Emirates, United States, Kuwait, Hong Kong and Malaysia, with 60.3, 46.1, 43.0, 38.8, and 38.3, respectively. On the other hand, the countries that consumed the least broiler meat were India, Indonesia, Philippines, China and Ukraine, with 1.8, 2.8, 7.5, 7.9, and 9.7 kilograms per person, respectively. The United States leads the world per capita in turkey meat consumption with 7.6 kilograms per person, while Canada and the European Union consume 4.4 and 3.9 kilograms per person, respectively (USDA-FAS, 2007). Based on these data, it could be hypothesized that economics and cultural factors could contribute to the consumption of poultry meat in these countries.

Broiler production worldwide was estimated to be over 61 million metric tons for the year 2007. Of this total, the United States was calculated to produce over 16.4 million metric tons (8.88 billion birds) for the period under review. The US poultry industry represents an industry valued at approximately $26.8 billion, when receipts of

broilers, turkeys and egg are combined (USDA-NASS, 2007). As consumers are becoming more educated on the nutritional status of food commodities, there is a growing trend for consumers to shift from red meat, which is often associated with health risks, to white meat. Thus, poultry meat consumption is projected to consistently increase in the future.

Salmonella Contamination of Poultry

The commercial poultry industry consists mainly of fully integrated commercial enterprises. Within this production system, there are multiple opportunities where poultry can become contaminated with *Salmonella*, ranging from breeder flocks, to the hatchery, to the point of human consumption. Several contamination routes have been documented for *Salmonella* contamination and can be characterized as either horizontal or vertical. Horizontally, *Salmonella* contamination is very extensive and by no means limited to the following list: hatchery (Byrd et al., 1999; Capita et al., 2003), feed (Jones and Richardson, 2004; Maciorowski et al., 2005), litter (Caldwell et al., 1994; Line, 2002), transport or live-haul (Corry et al., 2002; Slader et al., 2002), processing plant (Chambers et al., 1998; Hinton et al., 2004; Olsen et al., 2003), and retail market (Simmons et al., 2003). Within these sites of production and processing, potential sources of contamination for poultry also include wild birds, rodents, insects (Bailey et al., 2001), and farm workers (Molbak et al., 2006). Vertical transmission of *Salmonella* from the breeder flock to young chick has been previously reported (Liljebjelke et al., 2005). A list of *Salmonella* sources, serotypes, and prevalence percentage are shown in Table 2-2.

Table 2-2. Global prevalence of *Salmonella* serotypes isolated from poultry origin.

Country	Source	Percentage	*Salmonella* serotypes	References
Brazil	Poultry carcass	42.0	Enteritidis, Albany, Hadar, Indiana	Fuzihara et al. 2000
Canada	Chicken crop	4.3	Heidelberg, Hadar	Chambers, et al. 1998
Korea	Retail carcass	25.9	Enteritidis	Chang, 2000
Malaysia	Poultry carcass Intestinal content Litter	25.9 14.3 20.0	Enteritis, Muenchen, Kentucky, Blockley	Rusul, et al., 1996
Saudi Arabia	Processed chicken Eggs	42.9 0.06	Enteritidis, Virchow, Paratyphi B Var. Java	al-Nakhli, et al., 1999
UK	Broiler house	25.0	Hadar, Enteritidis, Indiana	Jorgensen, et al., 2002
USA	Broiler house Tray liner	42.0 12.1	Heidelberg, Kentucky	Byrd, et al., 1999
USA	Chicken Carcass	6.1	Thompson, Montevideo, Heidelberg	Bailey, et al., 2001
USA	Chicken Carcass	38.0	Data not available	Simmons, et al., 2003

Salmonella *in the Hatchery*

To keep pace with the approximate 9 billion broilers that are reared in the United States annually, multiplier breeder flocks are needed to produce the many billions of fertile eggs that are sent to commercial hatcheries for hatching. There is a growing concern among hatchery personnel over the high frequency of *Salmonella* contamination of these fertile eggs. Infected eggs have the potential to produce chicks that are *Salmonella*-positive (Vizzier-Thaxton et al., 2006). In a study reported by Cox et al. (1990), *Salmonella* was detected on 75.4% of hatchery samples. Similarly, a study of seven hatcheries concluded that 12.1% of tray liners were *Salmonella*-positive (Byrd et al., 1999). More recently, *Salmonella* was isolated from two hatcheries at frequency levels of 20% (Liljebjelke et al., 2005). The difference in *Salmonella* contamination could be attributed to the sampling method, sampling time, and geographical locations. Furthermore, it could be hypothesized that some hatcheries have better sanitation programs in place than others, which could explain why the *Salmonella* may vary among studies.

The ability of *Salmonella* to be transmitted from the hen to the progeny has been well documented. During ovulation the egg may be contaminated as it travels along the reproductive tract (Shivaprasad, 2000). In contrast, researchers have identified that semen from roosters may be a potential carrier for *Salmonella* during mating. Electron photomicrographs of chicken semen has revealed *Salmonella* present on the head, mid-piece and tail of spermatozoa (Vizzier-Thaxton et al., 2006). Therefore, infected sperm

cells can contaminate the eggs during fertilization, thus producing *Salmonella*-positive chicks.

Salmonella *in Feed*

There have been some debates whether or not feed is a potential source of *Salmonella* contamination for poultry. A study of two feed shipments to a turkey farm, Flock 1 and Flock 2, found *Salmonella* contamination rates of 9.1% and 18.8%, respectively (Hoover et al., 1997). Similarly, al-Nakhli and co-workers (1999) documented a 3.5% *Salmonella* recovery rate in poultry feed. Recently, a comprehensive study of three feed mills was conducted. The data showed that the mean *Salmonella* recovery was 8.8%, 4.8%, 3.2%, and 1.9% at the mixer, pellet mill, cooler, and loading area, respectively.

Interestingly, seasonal effects showed a higher isolation rate in April (4.6%) compared to (4.3%) August, however, the rates were not significantly different (Jones and Richardson, 2004). On the other hand, a Malaysian study found no feed samples positive for *Salmonella*. A drawback to the study was that only a few samples were collected and the feed samples were all pelleted. Pelleted feed has been know to reduce the *Salmonella* recovery rate (Rusul et al., 1996). Based on these observations feed could be a potential source of *Salmonella* contamination, but at very low frequency.

Grow-out

The grow-out facility has long been known to be a reservoir for *Salmonella* contamination. Modern commercial poultry houses are equipped with fully automated systems that control climatic conditions, feed, and water delivery for the birds.

With the advent of new technology the industry stocks the houses at higher placement densities (Reilly et al., 1988), when compared to decades ago. The increase in stocking density may result in poorer sanitary conditions, which becomes a favorable environment for disease outbreak at the farm level.

To indicate how the grow-out environment may influence *Salmonella* colonization in broilers, Bailey et al. (2002) reported a very high *Salmonella* colonization of 60% in a grow-out flock. One of the most comprehensive epidemiological investigations into the impact of live-production on *Salmonella* colonization in broilers was published by Bailey and coworkers (2001). This elaborate study consisted of taking approximately 8,740 independent samples from rearing houses of four integrators located in four different states in the US. Among the four integrators, *Salmonella* recovery rate overall ranged from 5.2% to 13.4%. *Salmonella* prevalence on the farm was highest in the fall (13.9%) and lowest in summer (5.4%). Byrd and colleagues (1999) reported *Salmonella* isolation rate of 42% from 196 poultry houses. Sampling methods can influence the recovery rate, which may provide some insight into the differences in the results of the various data. For example, surgical shoe covers improve detection rates six times compared to drag swab (McCrea et al., 2005). Therefore, *Salmonella* frequency data generated from drag swab could be higher in reality, but was not detected.

Feed Withdrawal and Transportation

Feed withdrawal prior to transportation to the processing plant is a standard practice in the poultry industry and it serves to reduce feed wastage, empty the crop and

GI tract, and reduce fecal deposit in crates (Hinton et al., 2000b). However, the negative impact of feed deprivation is the increase of pathogenic bacteria in the gastrointestinal tract (Hinton et al., 2000a). *Salmonella* prevalence in the crop before and after feed withdrawal has been well studied. Birds that are full fed have lower crop pH compared to feed-deprived birds. Similarly, birds with an 8-h feed withdrawal regime were colonized with *Salmonella* more than 3.8 times than the control (Corrier et al., 1999). These observations were also noted in a previous study (Ramirez et al., 1997).

As a preharvest treatment, the administration of lactose (Barnhart et al., 1999) and lactic acid (Byrd et al., 2001) have been evaluated for the ability to reduce *Salmonella* recovery subsequent to feed withdrawal. Providing lactose to birds in drinking water 5 to 11d before processing failed to reduce *Salmonella* colonization in a commercial environment (Barnhart et al., 1999). In contrast, the application of formic acid and lactic acid to birds reduced *Salmonella* Typhimurium in broilers compared to controls following feed withdrawal (Byrd et al., 2001).

Prior to processing, broilers are transported from the farm to the plant in crates. Transportation equipment, crates, and chicken catch crew personnel are known sources of *Salmonella* contamination (McCrea et al., 2005; Rigby et al., 1982). Between flocks, crates are not always cleaned and sanitized, thus representing an avenue for cross contamination. Sanitization of transport crates according to the manufactures recommendations has been shown to reduce *Salmonella* contamination (Corry et al., 2002). In contrast, disinfectant provided no advantage in eliminating *Campylobacter*

from crate wash water. It could be hypothesized that the efficacy of the disinfectant was lowered by the accumulation of fecal matter in the wash water (Slader et al., 2002).

Processing

Commercial poultry processing has been known for decades to be a source of *Salmonella* contamination (Dougherty, 1974; Hargis et al., 1995; Morris and Wells, 1970; Nde et al., 2006). Each stage of processing can be a potential environment for *Salmonella* contamination. Recent data provided evidence that 36% of broiler carcasses were *Salmonella*-positive (Bailey et al., 2002). The process of defeathering has been associated with a high incidence of *Salmonella* contamination. In one study, broilers were 7% positive at a pre-scald location and increased to 16% post-defeathering (Nde et al., 2006). The scalding tank and rubber fingers of feather pickers have been known to harbor pathogens (Clouser et al., 1995). Increased contamination within this area of processing could be associated with poor sanitation of the equipment (Campbell et al., 1984). Contrary to previous reports, the scald tank has shown demonstrated to reduce bacterial load on turkey carcasses by approximately 1.0 log CFU/g. The author suggested that scalding removes pathogens embedded in dirt and fecal droppings from the feathers (Goksoy et al., 2004).

The contribution to *Salmonella* prevalence by evisceration is well documented. Evisceration can damage the gastrointestinal tract, especially if feed is still present. During evisceration, the crop and ceca are subject to rupture and could contaminate the entire carcass (Byrd et al., 1998; Chambers et al., 1998). A study by Hargis and others (1995) reported a higher frequency of *Salmonella* in the crop (52%) than ceca (14.6%)

and that the crop was 86 times more likely to rupture and contaminate the carcass than the ceca. In Canada, the prevalence of *Salmonella* in the crop was very low (4.3%). The author suggested that the difference in results between both studies could be explained by the difference in temperatures in Texas and Canada (Chambers et al., 1998). A recent study of several pathogenic bacteria demonstrated that the crop is twice as likely to be positive for pathogens than the gizzard (Smith and Berrang, 2006).

There is supporting evidence to prove that the use of inside-outside bird washers (IOBW) on processing lines lowers pathogen loads on carcasses. There is some indication that *Campylobacter* rate was reduced following the application of multiple IOBW to carcasses (Smith et al., 2005). Furthermore, the addition of acidified sodium chlorite (ASC) to chill tanks reduced *Campylobacter* and *Salmonella* compared to IOBW. To improve quality control, it is more efficient to use antimicrobial treatment than IOBW (Kemp et al., 2001). To improve quality control, it is more efficient to use antimicrobial treatment than IOBW. In addition, the combination of both methods may further reduce pathogens. IOBW reduced *Salmonella* detection rate from 100% to 33.3% in artificially contaminated carcasses (Smith et al., 2005).

The chill tank also has been implicated as a major contributor to contamination of poultry carcasses (Hinton et al., 2004; McCrea et al., 2006). According to recent data, 10% of birds that entered the chill tank were contaminated with *Salmonella* and at post-chill carcasses were 16% positive (Nde et al., 2006). Nevertheless, other researchers found that chilling is an effective measure to reduce *Salmonella*, the data showed reduction from 52% to 13% in broiler carcasses subsequent to chilling (Mikolajczyk and

Radkowski, 2002). In addition, immerse chilling has reduced bacteria population by up to 3.44 $\log_{10}$ CFU/mL of rinsate (Hinton et al., 2004).

To ensure low contamination of the final product, processors are commonly employing intervention strategies at chilling. Application of chemical treatments such as chlorine, hydrogen peroxide, acetic acid, ozone (Vadhanasin et al., 2004), and trisodium phosphate (Bourassa et al., 2004) to chill tanks have shown favorable response in bacterial control. In a chill tank simulated experiment, carcasses were treated with three interventions; hydrogen peroxide, per acetic acid, and ozone, then compared to results in control chlorinated water. *Salmonella* prevalence among groups was as follows: chlorine, hydrogen peroxide, acetic acid, and ozone at rates of 22.7%, 16.0%, 5.0%, and 15.0%, respectively (Vadhanasin et al., 2004). The results suggested that chlorine was not very useful in lowering *Salmonella* contamination. The application of TSP to broiler carcasses showed that *Salmonella* was lowered at processing and on 7 d-old refrigerated carcasses (Bourassa et al., 2004). It may worth mentioning that some bacteria are resistant to chlorine and could remain dormant during chilling and could later reproduce during refrigeration (Hinton et al., 2004).

The retail market has been regarded as an outlet for *Salmonella* recovery. A study conducted in the United Kingdom discovered that 4.4% of fresh and 9.4% of frozen chicken carcasses were *Salmonella*-positive at retail. Retailers had 3.8 times higher *Salmonella* contamination compared to the butchers. The difference in results could be that 70% of retailers were sampled in comparison to 30% of the butchers

(Meldrum et al., 2005). A 20-Wk study in the United States testing 251 broilers carcasses, showed that 33.9% were *Salmonella*-positive at retail (Simmons et al., 2003).

Salmonella Typing Methods

Several methods of pathogen typing systems have been developed over the years. Typing systems are generally divided into two broad categories: phenotypic (conventional, traditional) and genotypic (molecular, PCR-based). Phenotypic systems include serotyping, phage typing, antibiotic resistance (R-type), biotyping, antibiogram, and bacteriocin (Cooke et al., 2007; Sader et al., 1995). Of the phenotypic methods, serotyping and phage typing are widely utilized to type *Salmonella*. Recently, multiple DNA-PCR-based methods have been employed to detect foodborne pathogens. Molecular-based methods involve the use of PFGE, random amplified polymorphic DNA (RAPD), ribotyping, amplified fragment length polymorphism (AFLP), REP-PCR (Cook et al., 1998; Olive and Bean, 1999; Versalovic et al., 1993), and DGGE (Muyzer et al., 1993,1998). Subsequent discussions of *Salmonella* typing in this review will focus mainly on serotyping, REP-PCR, and DGGE.

Currently, conventional techniques of foodborne pathogen detection are widely being practiced (Lin and Tsen, 1999). *Salmonella* detection includes subjecting the isolate to non-selective preenrichment, selective preenrichment, plating on selective media, and diagnostic agar (Jenikova et al., 2000; Whyte et al., 2002). Preenrichment provides the avenue for the organism to resuscitate from injury and to become acclimatized to the new environmental conditions (Hanes, 2003). Presumptive *Salmonella* undergo several biochemical tests on triple sugar iron (TSI) and lysine iron

agar (LIA) to confirm the organism to the genus *Salmonella* prior to serotyping (Hanes, 2003). It cannot be debated that conventional culture techniques are labor intensive, time consuming, expensive, non-sensitive, and non-specific (Bohaychuk et al., 2005; Jitrapakdee et al., 1995; Oliveira et al., 2003). On average, an estimated time span of 4 to 7 d is required to obtained a positive result, excluding the time for serotyping (Jin et al., 2004; Seo et al., 2003; Wang and Yeh, 2002).

Serotyping is normally performed in reference laboratories and requires large stocks of commercial antisera (Christensen et al., 2000b; Grimont et al., 2000). Currently, in the United States, *Salmonella* isolates have to be shipped to the National Veterinary Services Laboratory (NVSL) for serotyping, each isolate costs US $35 and could take up to one month to receive the confirmed serotype. Based on personal observations, serotyping is prone to errors. *Salmonella* isolates that are within the same serogroup at times could be difficult to differentiate. In one case, an isolate previously serotyped as *Salmonella* Derby was returned for serotyping to check reproducibility. The previously serotyped *S.* Derby isolate was confirmed to be *S.* Agona. Interestingly, both serotypes are within the same serogroup. However, using a PCR-based DNA fingerprinting technique the problem was resolved. In addition, some bacterial isolates are untypeable as previously observed in *E. coli* and were characterized to be rough and non-motile (Jonas et al., 2003). Over the years, researchers and laboratory personnel have been searching for alternative typing methods that are rapid, sensitive, reproducible, and inexpensive.

PCR-based molecular techniques have been widely accepted as an alterative to conventional methods in pathogens detection (Jitrapakdee et al., 1995; Johnson and Clabots, 2000). These techniques have become the gold standard for amplifying genomic DNA in modern research laboratories. Techniques such as DGGE, PFGE, REP-PCR, and real time-PCR are commonly used to discriminate among bacterial species, serotypes, and strains. DNA fingerprinting techniques have been known to be fast, sensitive, specific, reproducible, and less labor intensive in detecting foodborne pathogens. Nevertheless, each method has its own limitations. The results from pathogen detection by the above genotypic methods are available within a 24-h to 30-h period (Oliveira et al., 2003; Whyte et al., 2002). Molecular techniques have been well exploited in infection control and epidemiology environment (Johnson and Clabots, 2000).

Principle of Polymerase Chain Reaction

PCR is an enzymatic replication of DNA *in vitro* (Mullis and Faloona, 1987; Rashtchian, 1995). The technique was developed by Kary Mullis in 1986 while employed at Cetus Corporation (Erlich, 1999). PCR has revolutionized the field of molecular biology and has become a standard practice in modern laboratories (Arnheim, 1990; Rodriguez, 1997). There are thousands of publications available describing the use of PCR. PCR has been well exploited in the fields of pathogen detection, forensic studies, human medicine, and veterinary diagnostics (Ausubel et al., 1994; Jitrapakdee et al., 1995).

PCR involves the use of two complementary oligonucleotide primers, magnesium salt, DNA polymerase, deoxynucleotide triphosphates (dNTPs), DNA template, and buffer (Mullis, 1990a). DNA structure is double helix and contains four deoxynucleotide: deoxyadenylate (A), deoxythymidylate (T), deoxyguanylate (G), and deoxycytidylate (C) (Mullis, 1990b). Generally, the primers are about 20 base pairs in length and should consist of 50-60% G-C content (Baumforth et al., 1999). The dNTPs supply energy and the DNA bases for the production of the new DNA products (Baumforth et al., 1999). DNA polymerase is an enzyme isolated from a thermophilic bacterium, *Thermus aquaticus*, found in the hot spring and is relatively stable at high temperatures required for PCR. The heat stable enzyme increases reliability, precision, convenience, and reduces labor cost of the reaction (Bloch, 1991).

Currently, PCR amplification is fully automated and is performed in a thermal cycler. Enzymatic amplification of DNA involves three repetitive steps known as a cycle: denaturing, annealing, and extension (Mullis et al., 1986). Subsequent to each cycle, the DNA strands are doubled (2^n) and serve as the template for the next cycle. Theoretically, after 30 cycles (2^{30}) of amplification and with 100% efficiency, over one billion copies are expected to be generated (Erlich, 1989). DNA is denatured at high temperature (90-95 C) as the double helix unwound into two single strands. Excess or the lack of heat can reduce the yield of the PCR product by reducing the specificity of DNA polymerase (Saiki et al., 1988). Annealing temperature is dependent on the G-C content of the primers. Following denaturing, the temperature is lowered to 45-60 C and the primers hybridize to the complementary strands of DNA (Powledge, 2004). Primer

extension is usually done at 72 C. The DNA polymerase extends the primers along the target region to form new strands (Niemeyer, 1998; Powledge, 2004).

Denaturing Gradient Gel Electrophoresis

PCR-based DGGE has been well characterized in gut ecology studies (Donskey et al., 2003; Hume et al., 2003; Muyzer et al., 1998). DGGE is a culture-independent fingerprinting technique and has been reported to be reliable, rapid, cheap, and highly reproducible (Ercolini, 2004; Muyzer, 1999). DGGE was first introduced into gut ecology studies by Muyzer and coworkers (1993). Since the debut of DGGE in bacterial taxonomy, DGGE has gained considerable attention and several workers have been exploring the use of the technique in other areas of research. DGGE has been used in microbial typing and identification of bacteria in soil, clinical samples, insects, water isolates (Fromin et al., 2002), monitoring population shifts, evaluating extraction methods, cloning (Muyzer et al., 1998) and has recently emerged in food pathogen detection (Ercolini, 2004).

DGGE as applied to bacterial populations exploits ribosomal DNA (rDNA) fragments of the conserved region that lies next to the hypervariable V3 region of the 16S rDNA gene (Muyzer et al., 1993). Prokaryotes contain three genes on the rDNA codon: 5S rDNA, 16S rDNA and 23S rDNA (Chiu et al., 2005; Jensen et al., 1993). The internal spacer region (ISR) that is located between the 16S and 23S genes has been well documented in bacterial profiles (Christensen et al., 2000a). In addition, the ISR between the 16S and 23S rDNA as well as the 16S rDNA gene are well described in bacterial taxonomy (Chiu et al., 2005; Perez Luz et al., 1998). Bacteria ISR differ in length and

sequence and the variation allows for bacteria genera and species differentiation (Gurtler and Stanisich, 1996; Jensen et al., 1993). Studies involving the use of the 16S rDNA have shown that only about 10% of bacterial communities have been isolated and identified (Bjerrum et al., 2006).

DGGE is used to separate nucleic acid fragments that are identical in length, but of different nucleotide sequences (Muyzer et al., 1993; Wawer and Muyzer, 1995). DNA fragments are separated on polyacrylamide gel with a DNA denaturing gradient. The gradient environment is created by the application of urea and formamide to the polyacrylamide gel mixture. A 100% solution contains 7M urea and 40% formamide in water (Ercolini, 2004; Roelfsema and Peters, 2005). Two types of gradient gels are used for DGGE: perpendicular and parallel. In a perpendicular gradient, the gel has an increasing denaturing gradient that is perpendicular (90 degree angle) to the direction of the electrical field (Muyzer et al., 1998). A perpendicular gel can only accommodate one isolate and is typically used to observed melting characteristics of DNA and to establish the appropriate denaturing gradient range for future runs (Ercolini, 2004). On the other hand, parallel gradient gels have increasing gradient from the top to the bottom of the gel (Muyzer et al., 1998). In addition, parallel gradient gels a have smaller denaturing range, thus increasing separation of the DNA fragments. Several samples can be run on a parallel gradient gel. Parallel gradient gels are the more common in laboratories. DNA separation is done at temperature ranging from 55 to 60 C, however, 60 C is widely accepted (Ercolini, 2004).

Double stranded DNA migrates along the increasing denaturing gradient and separates or melts in a “discrete so-called melting domain” (Muyzer et al., 1998). As a result, DNA molecules with different nucleotide sequences and G-C content will migrate to different distances along the gel (Ercolini, 2004; Muyzer et al., 1998). The addition of a 40-50 GC-rich (GC-clamp) sequence to the 5’ end of one of the primers increases the temperature requirement of that fragment (Roelfsema and Peters, 2005). Complement bases in GC-clamps are held together by three hydrogen bonds in comparison to two hydrogen bonds in complement TA nucleotide complexes making them harder to denature (Nakatsu, 2007). Furthermore, the GC-clamp attachment prevents rapid and complete denaturation of the double helix into single stranded DNA, while the complementary sequences attached to the clamp will separate and restrict further migration (Muyzer and Smalla, 1998).

The different fragments detected on the gels correlate to the different bacterial species in the sample (Nakatsu, 2007; Teske et al., 1996).

DGGE like other PCR-based techniques has some drawbacks. DGGE only reliably separates PCR fragments that are less than 500 base pairs in length (Muyzer et al., 1998; Roelfsema and Peters, 2005). In addition, DNA fragments with different sequences could be problematic at times to separate due to co-migration of these fragments (Muyzer et al., 1998). According to Ercolini (2004), only a limited number of DNA fragments can be separated due to poor gel resolution. Similarly, DGGE has low sensitivity to microbes that are present in limited quantities (Muyzer, 1999).

Repetitive Extragenic Palindromic-PCR

According to Olive and Bean (1999), repetitive extragenic palindromic-PCR is steadily emerging as the premier DNA fingerprinting technique. REP-PCR, targets the conserved interspersed repetitive elements that are distributed throughout the genome of prokaryotic and eukaryotic organisms (Frye and Healy, 2006; Versalovic et al., 1991). In addition, REP-PCR has the discriminatory power to identify bacteria at the subspecies and strain levels (Beyer et al., 1998; Healy et al., 2005; Olive and Bean, 1999). One drawback to REP-PCR is that some bacterial strains may not have the required number of element repeats to provide high discriminatory power (Foley and Grant, 2007). Interestingly, REP-PCR has been demonstrated to have high discriminatory powers that are comparable to PFGE, ribotyping, RAPD, and sequencing (Frye and Healy, 2006; Olive and Bean, 1999; Scott et al., 2002). These observations were also noted previously in a study comparing PFGE and REP-PCR and reporting that PFGE generally may be more discriminatory. The authors disclosed that seven sets of isolates that were not differentiated by PFGE were identified using REP-PCR (Weigel et al., 2004). In addition, REP-PCR is more cost effective and requires less time than the other methods (Olive and Bean, 1999). PFGE has been endorsed by CDC as the primary method for DNA genotyping (Hunter et al., 2005) however, REP-PCR is comparable to PFGE, therefore, it could be considered as an alternative method for typing.

Three types of REP-PCR have been reported in molecular genotyping: repetitive extragenic palindromic, enterobacterial repetitive intergenic consensus (ERIC-PCR), and BOX-PCR (Seurinck et al., 2003). REP-PCR was first introduced as a molecular-based

DNA typing method in 1991 (Versalovic et al., 1991) and currently, the technique is fully automated (Healy et al., 2005). REP-PCR has less complexity, whereas ERIC has high sensitivity and is very useful in detecting contaminants resulting from improper DNA handling. On the other hand, BOX-PCR is superior to the other methods, creating more distinct fingerprinting patterns that can be used to trace host source in epidemiology studies (Scott et al., 2002). Of the three, REP-PCR and ERIC-PCR are the methods primarily used for genotyping (Frye and Healy, 2006). REP-PCR has a consensus sequence that is 38 base pairs in length in addition to a variable 5 base pairs in the stem loop of the palindrome structure. The sequence of ERIC-PCR is 126 base pairs and is also found in the extragenic regions (Koh-Luar et al., 1998; Olive and Bean, 1999; Versalovic et al., 1991). BOX-PCR has three subunits, BOX-A, BOX-B, and BOX-C, with nucleotide lengths of 59, 45, and 50, respectively. Interestingly, BOX-PCR shares no sequence relations with either REP-PCR or ERIC (Olive and Bean, 1999).

The conserved region that lies close to the repetitive extragenic palindromic regions differs according to size, thus, producing fragments of varying size and is evident via agarose gel electrophoresis (Foley and Grant, 2007). The fragment size provides a distinct fingerprinting profile for the organisms (Frye and Healy, 2006), which forms the basis for bands comparison (Foley and Grant, 2007).

Most recently, the manual REP-PCR method has been replaced with a fully automated system, commonly referred to as the DiversiLab system and several authors have provided a thorough review of the subject (Frye and Healy, 2006; Healy et al., 2005). Briefly, the inventors improved the reagent kit, replaced agarose with a

microfluidics chip and included online software for analysis of fragments (Frye and Healy, 2006). Furthermore, the time to separate and detect PCR product is reduce from 8 h in manual REP-PCR to 1 h. A high resolution digital camera that was previously needed has been replaced with an automated Agilent 2100 Bioanalyzer. Less biohazard waste from the ethidium bromide (<30 µL of 1.5 % acrylamide) has been incorporated into the chip. The DiversiLab system creates a customized report that was not available previously. Although the cost of the equipment and reagents may seem high, the savings are realized when the high cost of labor is factored into the equation (Healy et al., 2005).

A recent study by Healy and colleagues (2005) demonstrated that automated REP-PCR has high reproducibility among "multiple laboratories, personnel, laboratory equipment, various template DNA concentrations, multiple microfluidics instruments, and different culture conditions." Whereas, manual REP-PCR is plagued with poor reproducibility, is time consuming, and has poor separation on agarose gels.

CHAPTER III

MOLECULAR ANALYSIS OF *SALMONELLA* SEROTYPES AT DIFFERENT STAGES OF COMMERCIAL TURKEY PROCESSING

Introduction

Select serotypes of *Salmonella* collectively represent the predominant bacterial foodborne pathogens affecting humans in the United States. Poultry meat and eggs are considered to be a major vehicle for the transmission of *Salmonella* (Li and Mustapha, 2002; Vadhanasin et al., 2004). Approximately 10% of food related illness, totaling over 1.4 million cases annually, are associated with foodborne *Salmonella* infection in humans (Mead et al., 1999). Salmonellosis costs the US economy $2.4 billion per year when medical expenses, loss of productivity, and premature death is considered (USDA-ERS, 2005).

The Centers for Disease Control compiles a summary of the annual *Salmonella* serotypes isolated from human and nonhuman sources in the United States (CDC, 2004). Nonhuman sources of *Salmonella* serotypes provide information that is very useful in epidemiology studies to trace the origin of a known serotype found in human cases. In some instances, the most prevalent serotypes isolated from human and nonhuman sources are the same. In 2004, *S.* Typhimurium and *S.* Heidelberg were reported as the most prevalent serotypes found in nonhuman (clinical and non-clinical) and human cases (CDC, 2004). The CDC reported that the serotypes most frequently isolated from chickens were *S.* Heidelberg, *S.* Kentucky, *S.* Senftenberg, *S.* Enteritidis, and *S.*

Typhimurium. Whereas, in turkey the following serotypes were identified; *S.* Hadar, *S.* Senftenberg, *S*. Heidelberg, *S.* Muenster and *S.* Agona (CDC, 2004).

The use of PCR to amplify the spacer region between the 16S and the 23S rDNA is very useful in detecting, identifying, and differentiating bacteria isolates like *Salmonella* (Bakshi et al., 2002; Kostman et al., 1992; Nastasi and Mammina, 1995). The primers target the conserved region that lies next to the hypervariable V3 of the 16S rDNA (Muyzer et al., 1993). There are significant degrees of differences within the spacer region in regards to length and sequence. In addition, the wide range of variation among bacteria increases the probability for identification and typing between strains, species, and genera (Gurtler and Stanisich, 1996).

More stringent tests to detect foodborne pathogens like *Salmonella* must to be developed to safeguard the world's food supply. Recently, a study was conducted to evaluate the effect of PCR and conventional culture methods to identify *Salmonella* recovered from processed turkey carcasses. The results showed that PCR may improve identification by approximately 43% when compared to conventional methods. The author reported that the highest recovery was observed when PCR and conventional were combined (Whyte et al., 2002). However, PCR technique has proven to be very rapid, less labor intensive, very sensitive, reproducible, and more accurate than conventional culture methods. PCR presents an excellent diagnostic tool for fast screening and identification of *Salmonella* serotypes in epidemiology studies (Agarwal et al., 2002; Lagatolla et al., 1996; Pritchett et al., 2000).

Molecular-based PCR fingerprinting has become a gold standard for separation of genomic DNA in modern research environments. The use of denaturing gradient gel electrophoresis and polyacrylamide gel electrophoresis (PAGE) have been well documented (Ercolini, 2004; Hume et al., 2006; Muyzer et al., 1993). However, the use of DGGE as a diagnostic tool in identifying foodborne pathogens is still in its infancy (Ercolini, 2004). There are marked differences when separating DNA amplicons between the two techniques. PAGE separates PCR amplicons based on the relative molecular weight of the product. The larger the fragment size, the slower it travels thorough the acrylamide. However, DGGE separates PCR products of the same molecular size, but with different DNA sequences (Ercolini, 2004). Intact DNA is subjected to different concentrations of denaturant in the acrylamide gel and will eventually separate at different melting domains. When the desired domain is reached migration of the DNA will stop. As such, DNA with the same relative molecular weight, but different sequences, will migrate to different positions along the gel (Ercolini, 2004; Muyzer et al., 1998).

The addition of a 40-50-base pair GC-clamp to the 5' end of one of the primers can increase the stability of the double helix DNA by creating a higher melting domain (Ercolini, 2004; Muyzer et al., 1998). The added stability of the GC-clamp allows the newly formed amplicons to separate at their prescribed melting or denaturing levels in the gel, thus stopping the forward migration, but remaining connected at the still intact double-stranded clamp.

The objective of this experiment was to determine the utility of molecular techniques, specifically DGGE or PAGE, to distinguish individual *Salmonella* isolates recovered at various stages of processing in a commercial turkey processing facility.

Materials and Methods

Salmonella *Isolation*

The *Salmonella* isolates used in this study were from a previous project done in our laboratory. The samples were obtained from two commercial turkey processing plants (A and B), located in different geographical regions of the United States. The *Salmonella* isolates were recovered at different stages of processing. The sampling sites in Plant A were 1) post-scald, 2) pre-inside-outside bird wash (IOBW), 3) post-IOBW, and 4) post-chill, with n = 30, 44, 36, and 12, respectively. While Plant B sampling sites were: 1) pre-scald, 2) post-scald, 3) pre-IOBW, 4) post-IOBW and 5) post-chill, with n = 16, 54, 24, 35, and 24, respectively. The above *Salmonella*-positive isolates were stored on trypticase soy agar (TSA) at 4 C, prior to the start of the current study.

DNA Extraction

A loop (10 µL) of *Salmonella* from the TSA slants was repeatedly subcultured in tryptic soy broth (TSB) for 3 d at 37 C. On the third day of subculturing, 1.6 µL of *Salmonella* in TSB broth was placed into 0.4 µL of glycerol and stored at -80 C. In addition, *Salmonella* was streaked onto BGA (containing 25 µg/mL novobiocin) and grown overnight at 37 C for 18-24 h. A colony from each BGA plate was placed in 200 µL of TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and placed in boiling water for 15 min. The isolates were chilled and centrifuged at 8000 x *g* for 10 min. The supernate

from each isolate was removed and placed in a clean 1.5-mL centrifuge tube. DNA concentrations were standardized to 15 ng/µL (ND-1000, NanoDrop Technologies, Wilmington, DE), then stored at -20 C until needed for DNA amplification.

PCR Amplification

Polyacrylamide Gel Electrophoresis

PCR primers for PAGE were adapted from the protocol of (Bakshi et al., 2002). Two primers, forward G_1 5'-GAAGTCGTAACAAGG-3'and reverse L_1 5'-CAAGG-CATCCACCGT-3' (Integrated DNA Technologies, Coralville, IA), were used in the reaction. The mixture final volume was 50 µL and the constituents were as follows: 25.0 µL Jumpstart^{TM} PCR reaction Mix, (Sigma Chemical Company, St Louis, Mo.), 1µL of bovine serum albumin (BSA) (10 mg/mL), 1.25 µL each of G_1 and L_1 primer (50 ng/µL), 19.5 µL of PCR water (Sigma), and 2 µL of DNA template (15 ng/µL). Amplification of DNA was subjected to a 30-cycle program in a thermal cycler (PTC 200; MJ Research INC, Watertown, MA). The program was as follows: denaturation at 94 C for 1 min; annealing at 55 C for 2 min; and extension at 72 C for 2 min. The final cycle was followed by an additional 7 min at 72 C to complete partial polymerization (Bakshi et al., 2002). Prior to the start of the first cycle, the mixture was incubated at 94 C for 1 min to increase the final PCR product.

Denaturing Gradient Gel Electrophoresis

PCR-based DGGE was done according to the method previously reported by (Muyzer et al., 1993) with some modification (Hume et al., 2003). Two primers (50 pmol of each) were used; L_1 5'-CAAGGCATCCACCGT-3' and G_1 with a GC clamp,

5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGGAAGTCG-TAACAAGG– 3' (Integrated DNA Technologies) were mixed with commercial Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO). 1 µL bovine serum albumin (10 mg/mL), 2 µL of DNA template (15 ng/µL), and deionized water were added to make up a final 50-µL volume reaction. PCR of DNA was performed in a thermal cycler (PTC 200). The conditions were as follows: 1) denaturation at 94.9 C for 2 min; 2) subsequent denaturation at 94.0 C for 1 min; 3) annealing at 67.0 C for 45 s; –0.5 C per cycle [touchdown to minimize spurious by-products (Don et al., 1991; Wawer and Muyzer, 1995)]; 4) extension at 72.0 C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94 C for 1 min; 7) annealing at 58.0 C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72.0 C for 7 min; and 10) held at 4.0 C for the final stages (Hume et al., 2003).

Gel Electrophoresis

Polyacrylamide Gel Electrophoresis

In PAGE, a 5-µL aliquot of PCR product, combined with 2 µL of loading buffer (2x loading buffer, Promega, Madison, WI) was loaded per well. The marker well contained 4 µL (0.1µg/µL) of DNA ladder 100 bp (Ready load™, Invitrogen Life Technologies) mixed with 2 µL of 2x loading buffer (Promega). Separation of DNA fragments were carried out via PAGE, on a 5% polyacrylamide/bis gel (37.5:1) (Bio-Rad laboratories, Hercules, CA) in 1x TBE buffer (90 mM Tris, 90 mM Boric acid, and 2 mM EDTA, pH 8.4) at room temperature for 17 h at 250 V. Following electrophoresis, the gel was stained for 30 min using SYBR Green (1:10,000 dilution, Sigma).

Denaturing Gradient Gel Electrophoresis

The DNA fragments were resolved on an 8% (vol/vol) polyacrylamide-bisacrylamide gel (37.5:1) with a gradient of 35 to 45% (100% denaturing acrylamide; 7 *M* urea and 40% deionized formamide). Four microliters of PCR product were mixed with an equal volume of 2x loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 µL was placed in each sample well (20-well comb). Gel electrophoresis was carried out using 1x TAE (20 mM Tris, 10 mM sodium acetate, and 0.5 mM EDTA, pH 7.4) in a DCode Universal Mutation Detection System (Bio-Rad laboratories) at 59 C for 17 h at 60 V.

Imaging of Gel

The gel images (PAGE and DGGE) were digitalized (Alpha Imager®, Alpha Innotech Corporation, San Leandro, CA). The relatedness and dendrogram of fragment patterns were determined with Molecular Analysis Fingerprinting Software (version 1.610, Bio-Rad) based on the dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering.

Representative isolates from the different genotypic clusters were selected and serotyped for confirmation of serotype (NVSL). The isolates for serotyping were first grown on TSA with 5% sheep blood (BBL, Sparks, MD) at 37 C for 18-24 h, then a single colony was used to inoculate a TSA slant (Difco, Sparks, MD) and grown for 24 h at 37 C. A representative genotypic isolate from each plant was selected to be compared among all the different genotypes, thus creating a smaller dendrogram with fourteen genotypes, each representing a serotype.

Results

In the present study, *Salmonella* isolates were collected from two commercial processing plants (A and B) from two geographical locations in the United States. The sampling sites were similar for both plants except at Plant A, due to scheduling conflict, it was not possible to collect samples at a pre-scald sampling location.

Plant A

The distribution of the various *Salmonella* serotypes isolated from four sampling sites is presented in Table 3-1. A total of 122 isolates were determined to be *Salmonella*. There were four treatment sites namely: post-scald, pre-IOBW, post-IOBW, and post-chill, with n = 30, 44, 36, and 12 *Salmonella* isolates recovered per location, respectively. Of the total 122 *Salmonella* isolates, thirteen major clusters were observed on the dendrogram (not shown). As such, twenty-two (18%) representative isolates were selected from the various clusters to be serotyped. Within the 13 clusters, there were four different *Salmonella* serotypes identified: Derby, Hadar, Montevideo, and Senftenberg.

Approximately 83% of the total isolates recovered in Plant A were *Salmonella* Derby, clearly making it the most prevalent serotype throughout the plant (Table 3-1). At the post-scald location, predominant serotypes recovered were *S.* Derby, *S.* Hadar, and *S.* Senftenberg, with *S.* Derby being at the highest frequency. Post-scald recovery generated 25% (30/122) of the total isolates within the plant and all serotypes recovered in this plant were isolated at this location, with the exception of *S.* Montevideo. More isolates, 36% (44/122), were recovered at pre-IOBW than any other stage of processing.

Table 3-1. *Salmonella* serotypes isolated from Plant A turkey carcasses at various processing stages.

Salmonella	Treatments				
Serotype[1]	Post-scald	Pre-IOBW[2]	Post-IOBW	Post-chill	Total
Derby	13[3] (43.3)[4]	43 (97.7)	33 (91.7)	12 (100)	101 (82.7)
Hadar	9 (30.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (7.4)
Montevideo	0 (0.0)	1 (2.3)	3 (5.5)	0 (0.0)	4 (3.2)
Senftenberg	8 (26.7)	0 (0.0)	0 (0.0)	0 (0.0)	8 (6.6)
Total	30	44	36	12	122

[1]Conventional antibody serotyping
[2]IOBW-inside-outside bird wash
[3]Number of *Salmonella* positive isolates
[4]Numbers in parenthesis represent the percentage of the total serotypes per column

At this location, only two serotypes were found, S. Montevideo and *S.* Derby, of which *S.* Derby was the dominant serotype. Fewer *Salmonella* isolates were detected post-IOBW 30% (36/122) when compared to pre-IOBW. Post-chill was found to be the sampling location with the lowest isolation rate (10% or 12/122) as compared to all other locations. *Salmonella* Derby was the only serotype recovered at post-chill.

Plant B

The results presented in Table 3-2 represent *Salmonella* recovery frequency by serotype at the different stages of processing which were sampled in Plant B. There were five sampling sites in this facility: pre-scald, post-scald, pre-IOBW, post-IOBW, and post-chill, with n = 16, 54, 24, 35, and 24 *Salmonella* isolates recovered per site, respectively. Within this facility, a total of 153 isolates were recovered. Eighteen clusters were identified on the dendrogram (not shown) and 49 isolates were selected for serotyping. Ten *Salmonella* serotypes were identified in this plant including, Agona, Anatum, Brandenburg, Derby, Hadar, Meleagridis, Montevideo, Reading, Senftenberg, and Typhimurium.

In contrast to Plant A, *Salmonella* Typhimurium (39% or 59/153) was the most frequently isolated serotype in Plant B, and was the only serotype that was recovered at all sampling sites. At the pre-scald location, turkeys entered the plant with very low frequency of contamination (10% (16/153)) when compared to other locations. Four *Salmonella* serotypes were isolated pre-scald, including Derby, Typhimurium, Reading and Agona, with Derby isolation being at the highest frequency.

Table 3-2. *Salmonella* serotypes isolated from Plant B turkey carcasses at various processing stages.

Salmonella	Treatments					
Serotype[1]	Pre-scald	Post-scald	Pre-IOBW[2]	Post-IOBW	Post-chill	Total
Agona	2[3] (12.5)[4]	7 (13.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (5.9)
Anatum	0 (0.0)	1 (1.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.7)
Brandenburg	0 (0.0)	0 (0.0)	12 (50.0)	13 (37.1)	4 (16.7)	29 (18.9)
Derby	7 (43.8)	14 (25.9)	0 (0.0)	0 (0.0)	0 (0.0)	21 (13.7)
Hadar	0 (0.0)	1 (1.9)	2 (8.3)	6 (17.1)	8 (33.3)	17 (1.1)
Meleagridis	0 (0.0)	3 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)	3 (2.0)
Montevideo	0 (0.0)	4 (7.4)	0 (0.0)	0 (0.0)	0 (0.0)	4 (2.6)
Reading	3 (18.8)	4 (7.4)	1 (4.2)	0 (0.0)	0 (0.0)	8 (5.2)
Senftenberg	0 (0.0)	0 (0.0)	1 (4.2)	0 (0.0)	1 (4.2)	2 (2.3)
Typhimurium	4 (25.0)	20 (37.0)	8 (33.0)	16 (45.7)	11 (45.8)	59 (38.6)
Total	16	54	24	35	24	153

[1]Conventional antibody serotyping
[2]IOBW-inside-outside bird wash
[3]Number of *Salmonella* positive isolates
[4]Numbers in parenthesis represent the percentage of the total serotypes per column

The highest incidence of *Salmonella* recovery in this plant (35% (54/153)) was at post-scald location, with *S.* Typhimurium and *S.* Derby being the most frequent serotypes isolated. The serotypes *S.* Brandenburg and *S.* Senftenberg were not isolated post-scald. Interestingly, there was a shift in serotype recovery at the pre-IOBW sampling site, with *S.* Typhimurium and *S.* Brandenburg being the most common serotypes.

Correspondingly, *S*. Reading, *S*. Hadar and *S*. Senftenberg were isolated at low frequency. *Salmonella* serotype recovery post-IOBW increased by nearly 50% compared to pre-IOBW location. Despite the higher frequency, fewer serotypes were isolated post-IOBW, with recovery of only *S.* Typhimurium and *S.* Brandenburg occurring. At the post-chill site, the level of contamination was lower than post-IOBW, and four serotypes were isolated: *S.* Typhimurium, *S.* Hadar, *S.* Brandenburg, and *S.* Senftenberg. The most prevalent serotypes post-chill were *S.* Typhimurium (46%) and *S.* Hadar (33 %).

Cluster Analysis

Polyacrylamide Gel Electrophoresis

The dendrogram of PAGE genotypes detected (Figure 3-1) includes representative *Salmonella* serotype isolates found in both plants. Prior to the generation of the final dendrogram (Figure 3-1), separate dendrograms for Plant A and B were created (not shown). Plant A sampling resulted in the isolation of 122 distinct *Salmonella* isolates, of which four unique PAGE genotypes were identified. Plant B sampling generated the recovery of 153 *Salmonella* isolates, and subsequent analysis revealed ten genotypes present.

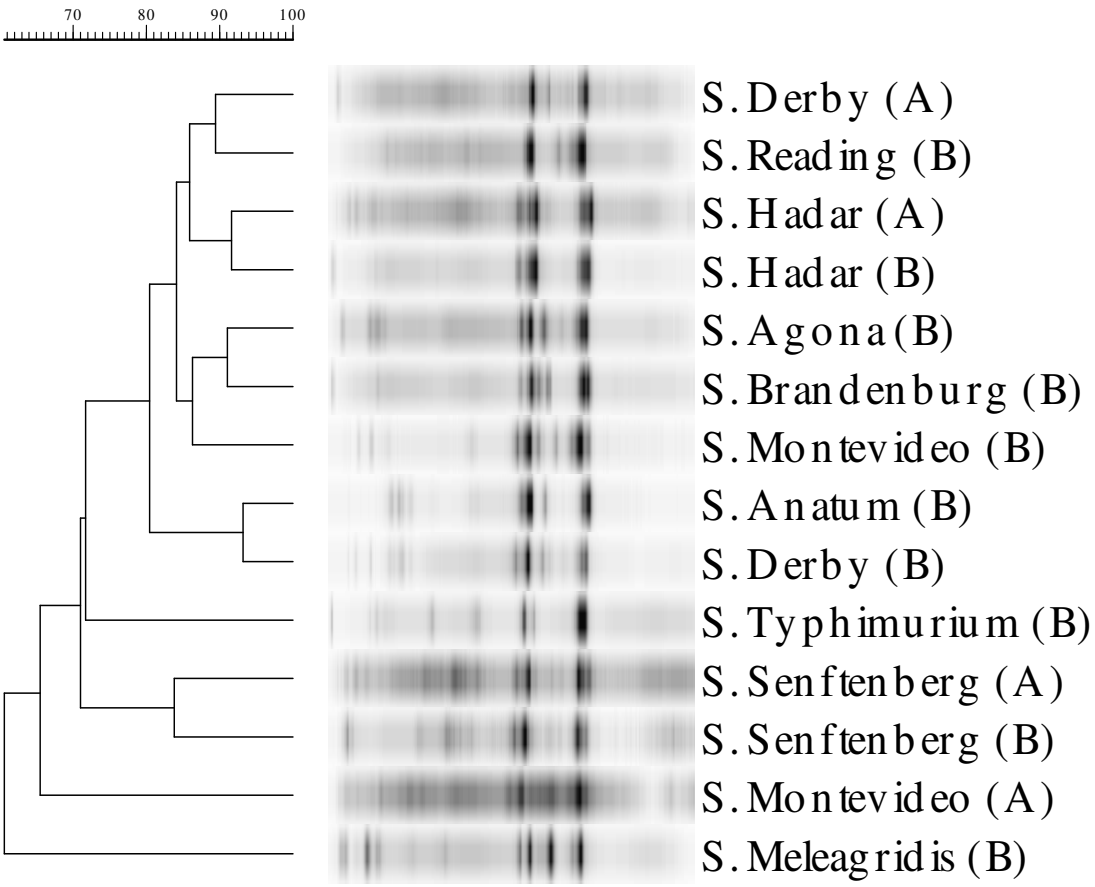

Figure 3-1. Dendrogram of polyacrylamide gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* serotypes recovered from two turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007).

To reduce error, multiple isolates were selected from each genotype for confirmation of serotypes and validity of the groupings on the PAGE dendrograms.

The results presented in Figure 3-1 represent the percentage similarity coefficient distribution among *Salmonella* isolates evaluated between Plant A and B. The dendrogram was created from representative isolates from each serotype from both plants. A total of fourteen representative isolates from twelve genotypes were examined and overall all the genotypes had a 59.9% similarity coefficient (SC). *S.* Derby (A), and *S.* Reading (B), showed 89.4% SC, whereas, the two genotypes of *S.* Hadar were grouped together with 91.6% SC. Additionally, genotypes for *S.* Agona, *S.* Brandenburg, and *S.* Montevideo showed 86.3% SC. The banding patterns of *S.* Anatum, *S.* Derby (B), and *S.* Typhimurium (B) displayed 71.7% SC. The two genotypes of *S.* Senftenberg had 83.8% SC, whereas *S.* Montevideo was different (71.0%) from all previous serotypes. In addition, *S.* Meleagridis was 59.9% SC also distinct from all other genotypes. All genotypes displayed similar primary bands between 400 and 600 base-pair and other secondary bands that were useful in discrimination among different PAGE genotypes.

Denaturing Gradient Gel Electrophoresis

The *Salmonella* isolates previously presented in PAGE comparisons were also used for DGGE analysis (Figure 3-2). Serotypes denoted by (A or B) were recovered from two different geographical locations. As previously mentioned there were a total of 10 serotypes recovered throughout the two processing plants.

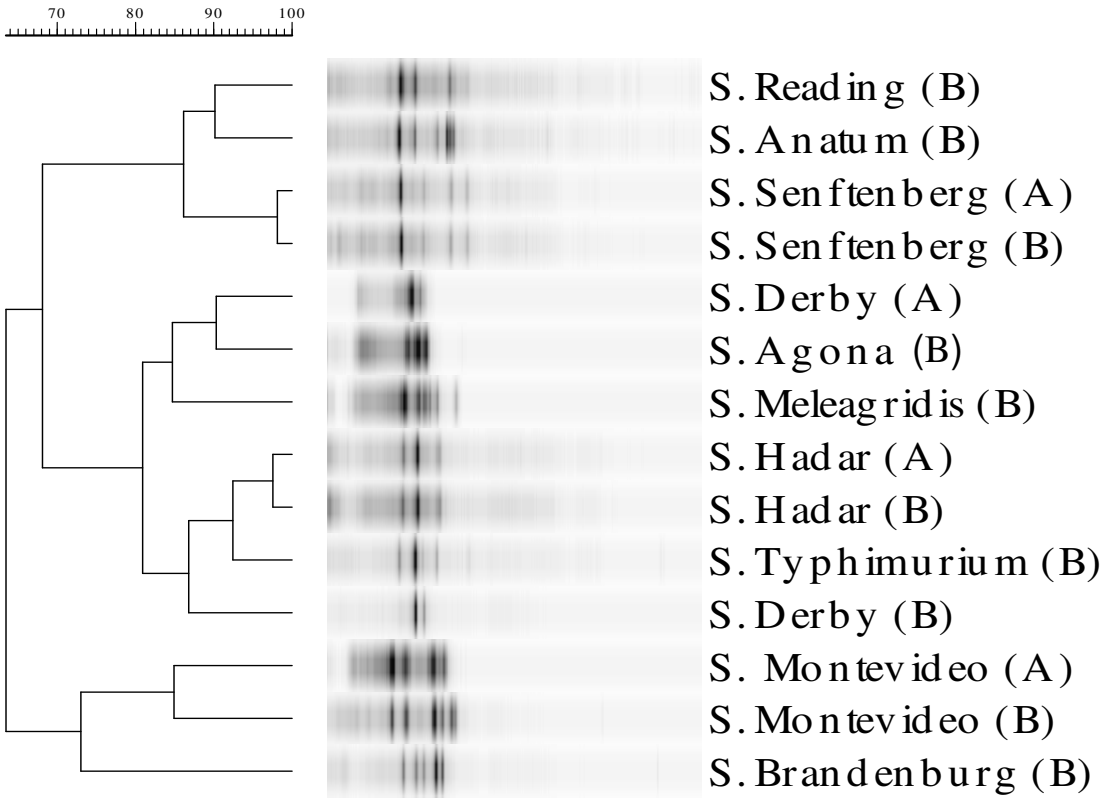

Figure 3-2. Dendrogram of denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* serotypes recovered from two turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007).

DGGE patterns for *Salmonella* Reading and Anatum had 90.1% SC, whereas, both Senftenberg isolates were likely identical (98.1% SC). S. Agona and S. Derby (A) had SC of 90.3%, while *S*. Meleagridis was distinct from that group due to an 84.7% SC. The *S*. Hadar genotypes were highly related (97.6% SC), but were slightly different (92.4% SC) from *S*. Typhimurium. *S*. Derby had 86.8% SC with the other genotypes within the cluster. The two *S*. Montevideo genotypes were isolated from two different geographical locations (Plants A or B) and were genotypically related (89.7 % SC). Both *S*. Montevideo isolates, however, were very different from *S*. Brandenburg due to only a 73.0% SC.

Discussion

These findings reveal that *Salmonella* serotypes isolated during commercial turkey processing may vary depending upon geographical (plant) location and within each plant. A previous study conducted in Malaysia of poultry processing plants demonstrated that different serotypes can be restricted geographically by region. For example, *S.* Blockley was isolated throughout the country, whereas, *S.* Enteritidis and S. Kentucky were recovered mainly in the central and region areas, respectively (Rusul et al., 1996). Other factors such as flock population, plant sanitation, age at slaughter, sampling method, season, and management have been shown to influence bacterial recovery in processing plants (Antunes et al., 2003). Only four serotypes from the current study were isolated from Plant A, while Plant B sampling resulted in the recovery of ten distinct *Salmonella* serotypes. *S.* Typhimurium was the most common serotype detected in Plant B, whereas, in Plant A the serotype isolated most prevalently

was *S.* Derby. This study is somewhat in agreement with several reports which demonstrated that the most common serotypes isolated from poultry were *S.* Enteritidis, *S.* Hadar, and *S.* Typhimurium (Leon-Velarde et al., 2004; Uyttendaele et al., 1998). Most recently, CDC published their annual summary on *Salmonella recovery from human and non-human sources*. According to the report, the six serotypes most frequently isolated from human sources were *S.* Typhimurium (19.2%), *S.* Enteritidis (14.1%), *S.* Newport (9.3%), *S.* Javiana (5.0%), *S.* Heidelberg (4.9%), and *S.* Montevideo (2.4%) (CDC, 2004). In our investigation, several *Salmonella* serotypes were present at different stages of processing. The differences in location may be a contributing factor in determining which serotype is more prevalent. *S.* Derby was the most common serotype isolated at Plant A, whereas at Plant B it was *S.* Typhimurium.

The use of PCR to amplify the variable region between 16S and 23S rDNA has been previously reported (Bakshi et al., 2002). Currently, several researches are manipulating the 16S and 23S rDNA to discriminate among bacterial species which makes this characterization useful in epidemiology studies (Jensen et al., 1993). PCR-based DGGE molecular fingerprinting technique is more sensitive and reproducible than the standard PAGE; however, it takes much more time to complete. The band patterns were more distinguishable in DGGE than PAGE. The band patterns for corresponding serotypes grouped as very similar in DGGE had higher similarity coefficients than related PAGE groupings. In the current study, band pattern comparison uncovered errors in the serotyping of some of the isolates when the fingerprinting profiles were not the same, even though they were reported as the same serotypes. Key features revealing this

apparent error in standard serotyping were the distinctive differences in the DGGE genotypic patterns in some comparisons of isolates reported as the same serotype. The anomalous isolates were returned for serotyping to check the credibility of the system. In some cases, the erroneously identified serotypes sent back for reexamination were returned from the diagnostic laboratory with an entirely different, and yet still mistaken serotyping. When errors were recognized from antigenic serotyping, analyzing the DNA bands profile was the method of choice to determine the correct serotype. Antigenic traits preventing some isolates from being correctly identified may be indicative of a limited fallibility of the serotyping scheme. However, the potential limited fallibility of the serotyping scheme may be more indicative of antigenic features of some serotypic strains that carry epitopes conveying multiple serotypic identities.

Salmonella serotypes have prominent bands on DGGE gels that are unique to most serotypes and could be classified as primary bands. However, there are secondary bands that provided the main discriminatory tool in distinguishing among the various serotypes (Bakshi et al., 2002). The results from the current study showed that *Salmonella* isolates of the same serotypes, but from different geographical locations in the United States, may differ in DGGE and PAGE band profile. In addition, other factors such as gel alignment and band intensity could affect the genotypic analysis. Evidence of these liabilities was observed among the *Salmonella* serotypes of Derby and Montevideo in Figures 3-1 and 3-2. *Salmonella* Senftenberg isolates, although having very similar banding profiles, had only an 83.8 % similarity coefficient. The reason for this mishap could be that the banding pattern for one of the isolates was darker than the

others, and the analytical program interpreted the patterns as being different. As noted in Figure 3-2, *Salmonella* Typhimurium appears to be similar to Hadar, but a slightly faded band associated with Typhimurium could have caused the mismatch. It is not always possible to run all isolates on the same gel, or run all isolates at the same time. This restriction could be very problematic with regards to gel alignment in the molecular fingerprinting program. Another point worth mentioning is the necessity for all the DNA to be extracted within the same time frame and, when possible, to use the PCR mixture from one batch. Such standardization can increase reproducibility by up to 100% (Garaizar et al., 2000).

The use of a molecular fingerprinting technique such as DGGE could be considered as an additional resource to confirm conventional bacterial serotyping based upon serology. DGGE is a reliable, accurate, reproducible, and inexpensive technique. In a pandemic scenario, it is necessary to characterize the pathogenic agent quickly, which makes it much easier to identify the best treatment for the affected victims. Therefore, having rapid results would be very economical.

Digitalizing of images increases the ability to synchronize collaboration between scientists from different laboratories across various regions of the world when working on a possible cure for an outbreak. Creating a library or genotypic data base of *Salmonella* serotypes would allow for fast identification of unknown serotypes. An added feature of a genotypic approach to *Salmonella* serotyping is reduced cost when numerous isolates are collected during an outbreak and have to be identified. However, more research of this nature is needed to fully substantiate such claims.

CHAPTER IV

EVALUATION OF REP-PCR AND DENATURING GRADIENT GEL ELECTROPHORESIS IN IDENTIFYING *SALMONELLA* SEROTYPES ISOLATED FROM PROCESSED TURKEYS

Introduction

Foodborne *Salmonella* infections represent a very significant threat to human health both within the United States and worldwide (Chang, 2000; Jorgensen et al., 2002; Liljebjelke et al., 2005). Globally, it is estimated that there are over 21.6 million cases of typhoid *Salmonella* documented annually (Crump et al., 2004). Nontyphoidal *Salmonella* infections are clearly more common, linked to over 1.3 billion cases and approximately 3 million deaths annually (Pang et al., 1995). In the United States, between 1996 to 1999, foodborne illnesses from *S.* Typhi were estimated to be fewer than 700 cases. Of these, 492 persons were hospitalized and 3 deaths occurred. Similar to global estimates, nontyphoidal *Salmonella* infection in the United States is estimated to result in 1.34 million cases, of which 16,430 persons were hospitalized, and resulting in 553 deaths reported (Mead et al., 1999). Annually, salmonellosis cost US $2.4 billion, resulting from medical cost, loss of productivity, and premature deaths (USDA-ERS, 2005).

Salmonella has been frequently reported in the products of plants and animals and poultry meat and eggs are considered to be a major vehicle for the transmission of *Salmonella* to humans (Capita et al., 2003; Li and Mustapha, 2002; Vadhanasin et al.,

2004). Several routes for contamination among commercial poultry have been estbablished, including the hatchery (Byrd et al., 1999), grow-out farms (Bailey et al., 2001; Caldwell et al., 1994), feed (Maciorowski et al., 2005), pre-processing transport or live-haul (Slader et al., 2002), processing (Corry et al., 2002), and breeder flocks (Liljebjelke et al., 2005).

Consumers are getting more health conscious with regards to food choices (Guo et al., 1999). The media could be credited for keeping consumers informed about foodborne pathogen outbreaks and the recalls of meats and products. Recalls are very costly to the shareholders of the poultry industry. As a quality control measure, several intervention strategies have been employed by commercial integrators, especially at the processing facility with the hope of reducing pathogens on the final product. Hence, the food industry is continuously exploring new pathogen detection methods that need to be inexpensive, fast, and reliable, to augment food safety strategies (Guo et al., 1999).

Conventional methods of testing foodborne pathogens are laborious, non-sensitive, slow, and often unreliable. Alternatively, DNA fingerprinting techniques have been shown to be fast, sensitive, specific, highly reproducible, and less labor intensive than conventional methodologies (Oliveira et al., 2003; Whyte et al., 2002). Aside from the demonstrated positives, each method has its own limitations (Agarwal et al., 2002). Molecular techniques have been widely accepted as the alternative to conventional methods in many research settings (Jitrapakdee et al., 1995; Johnson and Clabots, 2000). PCR-based techniques such as DGGE (Ercolini, 2004), PFGE (Oliveira et al., 2003; Whyte et al., 2002), REP-PCR (Jonas et al., 2003), and real time-PCR (Hein et al., 2006)

are some of the current methods used to discriminate among bacterial species, serotypes, and strains. The present study will focus on comparing DGGE and REP-PCR.

Muyzer and co-workers (1993) were the first to apply DGGE to microbial gut ecology studies. The primers targets the conserved region that lies next to the hypervariable V3 region of the 16S rDNA (Hume et al., 2003; Muyzer et al., 1993). The internal spacer region between the 16S rDNA and the 23S rDNA has been well exploited in prokaryotic organism such as *Salmonella* (Chiu et al., 2005). DGGE separates DNA fragments that are identical in length, but have different nucleotide sequences (Muyzer et al., 1993). Double stranded DNA migrate along the increasing denaturing gradient and melts in a "discrete so-called melting domain" (Muyzer et al., 1998). As the domain approaches the lowest temperature, the double helix partially melts and migration ceases. As a result, DNA molecules with different nucleotide sequences will migrate different distances along the gel (Ercolini, 2004; Muyzer et al., 1998; Muyzer et al., 1993). The addition of a 40-50 GC rich (GC-clamp) sequence to the 5' end of one of the primer increases the temperature requirement of that fragment (Roelfsema and Peters, 2005).

REP-PCR targets the highly conserved, interspersed, repetitive elements found at several sites within the eukaryotic and prokaryotic genome (Frye and Healy, 2006; Healy et al., 2005). The conserved region that lies close to the repeated elements differs according to size, thus, producing fragments of varying length, evident via agarose gel-electrophoresis (Foley and Grant, 2007). The fragment size provides a distinct fingerprinting profile for the organism (Frye and Healy, 2006), which forms the basis for band comparison (Foley and Grant, 2007). REP-PCR has the discriminatory power to

identify bacteria (*Bacillus subtilis*, *Batonella*, *E. coli*, and *Salmonella*) at the subspecies and strain level (Beyer et al., 1998; Healy et al., 2005; Olive and Bean, 1999).

Recently, manual REP-PCR has been replaced by an automated DiversiLab system. The DiversiLab system separates PCR amplicons on polyacrylamide microfluidics chips and a web-based program is used to create customized output (Frye and Healy, 2006; Healy et al., 2005). The system has been reported to be very time efficient and highly reproducible among laboratory technicians, microfluidics chips, DNA concentrations, laboratory equipment, and different culture conditions (Healy et al., 2005).

To the best of the author's knowledge, only a limited number of studies have compared DGGE and REP-PCR in characterizing foodborne pathogens. Most often, REP-PCR or DGGE are evaluated against PFGE, and both techniques have shown favorable response as an alternative method for genotyping. Therefore, there is a need to explore the discriminatory powers of REP-PCR and DGGE in pathogen detection and characterization. The objective of this experiment was to compare the use of automated REP-PCR and DGGE as potential diagnostic tools for identifying *Salmonella* serotypes.

Materials and Methods

Fifty-four *Salmonella* isolates collected from two turkey processing facilities (A and B) were used for this study. The serotypes present among these isolates were Brandenburg, Derby, Hadar, and Typhimurium (n = 6, 21, 12, and 15, respectively). All isolates were previously typed at the (USDA-APHIS) NVSL in Ames, Iowa. After their

initial isolation, all isolates were stored at -80 C in tryptic soy broth (TSB) containing 20% (vol/vol) glycerol until needed in this investigation.

DNA Extraction

Approximately ten microliters from the frozen stock culture were streaked onto BGA supplemented with 25 µg/mL novobiocin. BGA plates were incubated at 37 C for 18-24 h. Bacterial colonies from each plate were used for DGGE and REP-PCR DNA extraction.

Denaturing Gradient Gel Electrophoresis

A colony for typing by DGGE was chosen from each BGA plate was placed in 200 µL of sterile TE buffer (10 mM Tris/1 mM EDTA, pH 8.0) and the bacterial cells were lysed by heating in a boiling water bath for 15 min. The isolates were chilled and centrifuged at 10,000 x *g* for 10 min to separate out cellular particles, and the supernatant removed. Genomic DNA concentrations were measured spectrophotometrically (Nanodrop ND-1000, NanoDrop Technologies, Wilmington, DE) standardized to 15 ng/µL and stored at -20 C until needed for DNA amplification.

Repetitive Extragenic Palindromic-PCR

Approximately 2 µL (loop) of bacterial cells were used for DNA extraction for REP-PCR. The extraction was performed by exposing the cells to microbead beating, following the protocol of MoBio Ultraclean™ Microbial DNA Isolation Kit (Mo Bio Laboratory, Inc., CA) according to the manufactures recommendations. Following extraction, DNA was measured spectrophotometrically (ND-1000, NanoDrop

Technologies, Wilmington, DE) and adjusted to 25 ng/µL and then stored at -20 C prior to amplification.

PCR Amplification

Denaturing Gradient Gel Electrophoresis

The primers used were previously reported by (Bakshi et al., 2002) with slight modification. The amplification of the target region was achieved using two primers (50 pmol of each): forward G_1 5' GAAGTCGTAACAAGG-3', and reverse L_1 5'-CAAGGCATCCACCGT-3' (Integrated DNA Technologies). A GC-rich 30-base clamp 5'- CGCCCGCCGCGCGCGGCGGGCGGGGGGGCGGGG-3' was attached to the 5' end of the G_1 primer. The primers were combined with a commercial Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO). 1 µL bovine serum albumin (10 mg/mL), 2 µL of DNA template (15 ng/µL), and deionized water were added to make a final 50-µL reaction volume. PCR of DNA was performed in a PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA). The PCR protocol was adapted from a previous study (Hume et al., 2003), 1) initial denaturing at 94.9 C for 2 min; 2) subsequent denaturation at 94 C for 1 min; 3) annealing at 67 C for 45 s; –0.5 C per cycle [touchdown to minimize spurious by-products (Don et al., 1991; Wawer and Muyzer, 1995)]; 4) extension at 72 C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94 C for 1 min; 7) annealing at 58 C for 45 s; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72 C for 7 min; and 10) held at 4 C for the final stages.

Repetitive Extragenic Palindromic-PCR

Amplification was done using the DiversiLab fingerprint kit and following the manufacture's recommendations. Briefly, a master mix containing 18 µL REP-PCR MM1, 2.5 µL geneAmp 10x PCR buffer (Applied Biosystems, Foster City, Calif.), 2 µL Primer mix LL, 0.5 µL amplitaq DNA polymerase, and 2 µL (50 ng) DNA were added to a final volume of 25 µL. Amplification of DNA was performed in a PTC200 themocycler. The PCR conditions were initial denaturation at 94 C for 2 min, subsequently, a 35 cycles program of denaturing at 94 C for 30 s; annealing at 45 C for 30 s; and extension at 70 C for 90 s. Last, a single cycle for 3 min at 70 C was added to the final cycle.

Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis

DGGE was performed according to the method previously reported (Muyzer et al., 1993) with some modification (Hume et al., 2003). The PCR products were separated on an 8% (vol/vol) polyacrylamide-bisacrylamide gel (37.5:1) with a denaturing gradient of 35 to 45% (100% denaturing acrylamide; 7 *M* urea and 40% deionized formamide). Four microliters of PCR amplicons were mixed with an equal volume of 2x loading buffer [0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 µL was placed in each sample well (20-well comb). Gel electrophoresis was performed in a DCode Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA) using 1x TAE buffer (20 mM Tris, 10 mM sodium acetate, and 0.5 mM EDTA, pH 7.4) and ran at 59 C for 17 h at 60 V

(Hume et al., 2003). Following electrophoresis, the gels were stained using SYBR Green (1:10,000 dilution) in 1x TAE buffer for 30 minutes and destained using distilled water.

Repetitive Extragenic Palindromic-PCR

DNA fragments were separated on a 1.5% acrylamide gel microfluidics chip following the instructions of the manufacturer (Mo Bio Laboratory, Inc.). Briefly, 5 µL of DNA marker was added to each of twelve wells and also to the ladder well on the microfluidics chip (LabChip Device, Caliper Technologies, Inc.). Next, 1 µL of PCR product was added to the same wells. Finally, the microfluidics chip was vortex for 1 min, then placed for approximately 1 h in a model B 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA) for PCR fragment separation (Healy et al., 2005).

DNA Fingerprint Analysis

Denaturing Gradient Gel Electrophoresis

The DGGE gel images were digitalized (Alpha Imager®, Alpha Innotech Corporation, San Leandro, CA) and DNA fingerprint patterns were analyzed and a dendrogram was generated using the Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories), which operates according to the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering.

Repetitive Extragenic Palindromic-PCR

DNA analysis was performed using the DiversiLab software (version 2.1.66). The software created customized reports, including dendrogram, electropherograms, virtual gel images, and scatter plots (Healy et al., 2005). A DNA fingerprint profile of

the each lane was compared pairwise against all the other lanes. Pearson correlation coefficient was used to determine the percentage of similarity among the different bands patterns and UPGMA was used to create the dendrogram of the different clusters (Healy et al., 2005; Johnson and Clabots, 2000).

Results

Denaturing Gradient Gel Electrophoresis

Figure 4-1 represents the dendrogram of fifty-four *Salmonella* isolates from commercial turkey processing facilities located in two distinct geographical locations within the United States. The processing plants from which the isolates were recovered are labeled as Plant A and Plant B. The 54 *Salmonella* isolates consisted of four distinct serotypes: Brandenburg, Derby, Hadar, and Typhimurium. At 90% similarity coefficient (SC), there were five genotypic clusters observed among the isolates. *S.* Hadar contained twelve isolates and displayed genetic similarity of 95.4%. All the *S.* Typhimurium isolates were collected within Plant B and showed a consistent band pattern with 97.6% SC. The six *S.* Brandenburg DNA profiles were genetically identical at 99.2% SC. The two fingerprint patterns of the *S.* Derby isolates were very diverse and displayed unrelated profiles. All the *S*. Derby isolates from Plant B along with one isolate from Plant A exhibited a 99.0% SC. Similarly, the remaining thirteen *S.* Derby from Plant A were 98.0% related. Overall, the relatedness of *S.* Derby between the two plants was heterogeneous and exhibited only 67.7% similarity coefficient.

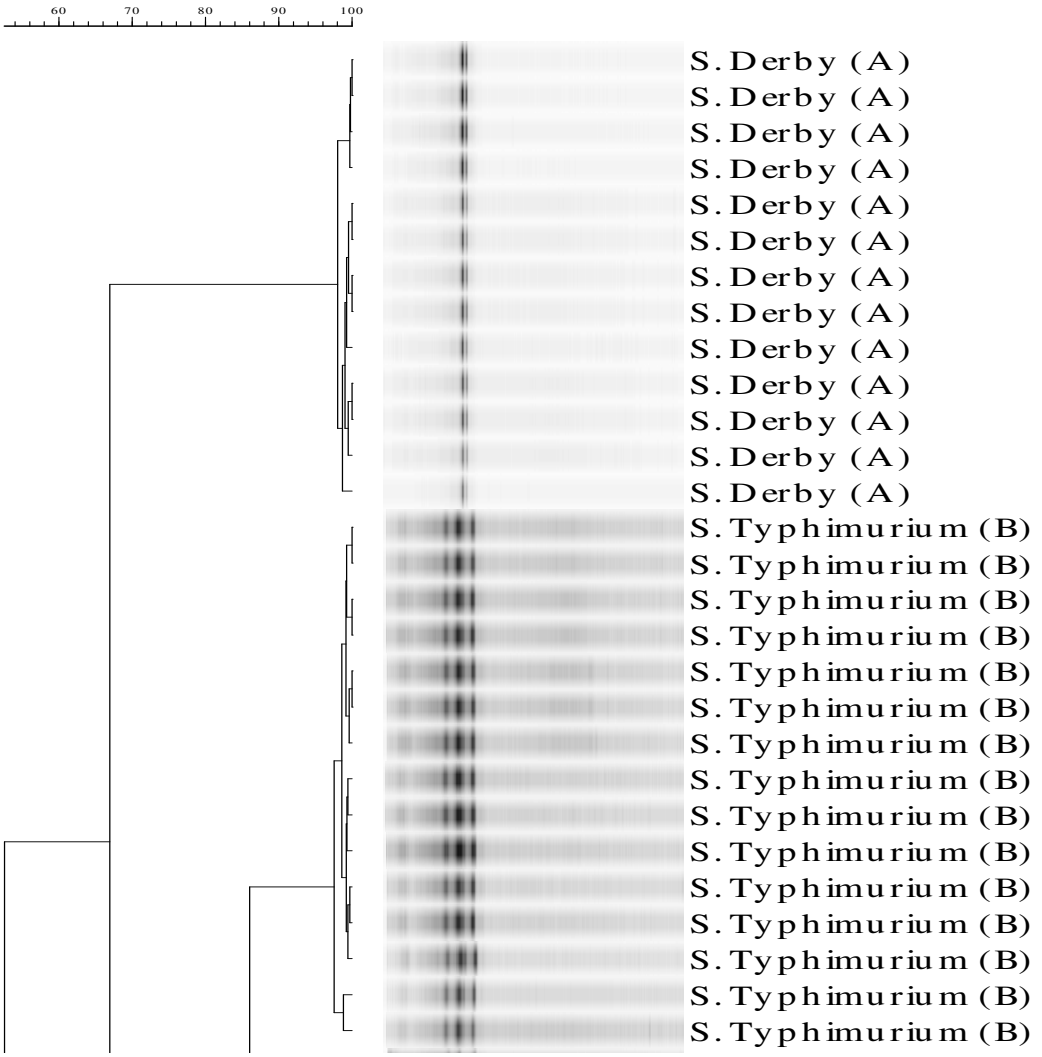

Figure 4-1. Dendrogram shows the denaturing gradient gel electrophoresis DNA profiles (16-23S rDNA) of fifty-four *Salmonella* isolates recovered from two turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007).

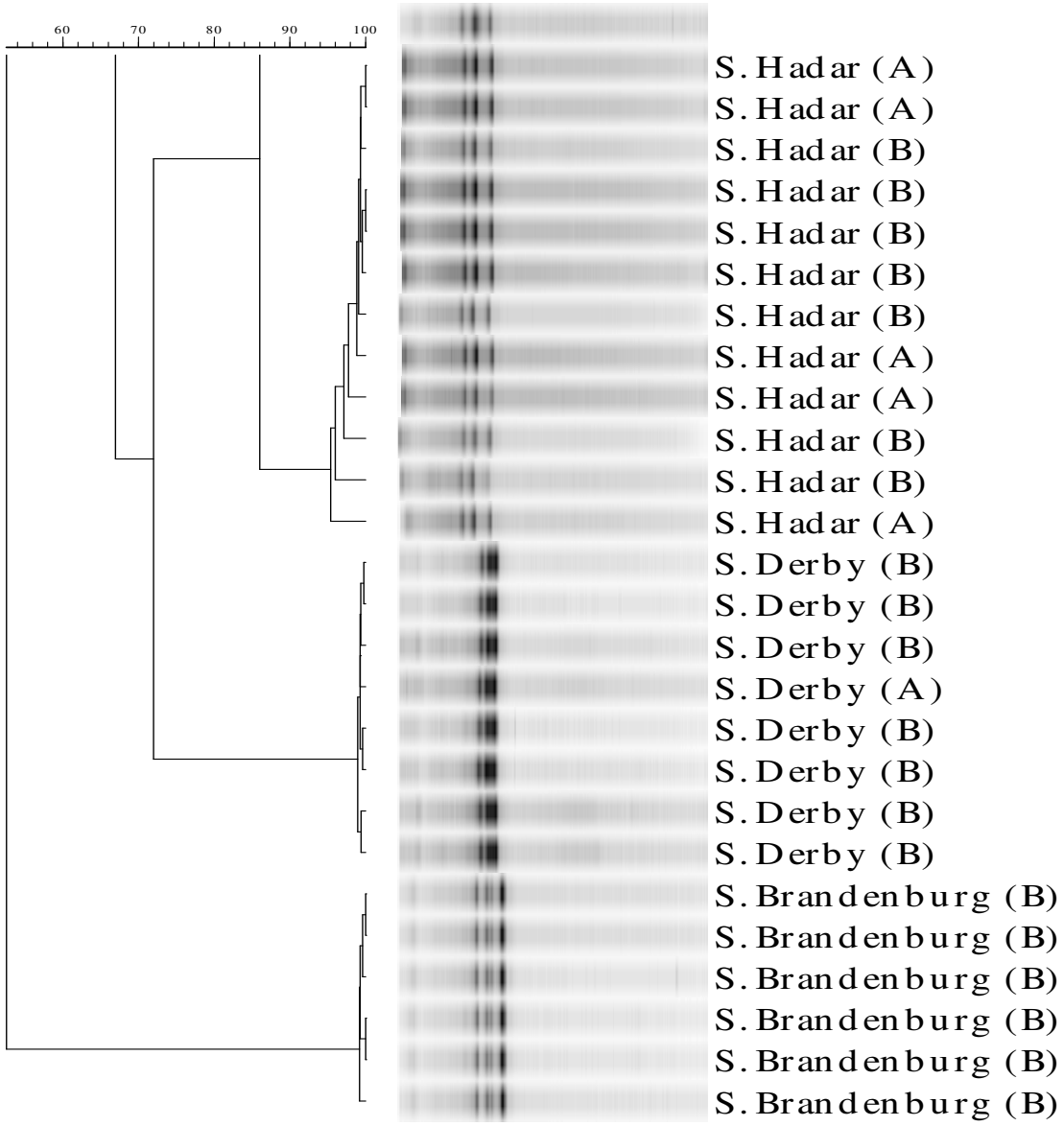

Figure 4-1 Continued

Repetitive Extragenic Palindromic-PCR

DNA fingerprinting profiles for the fifty-four isolates subjected to REP-PCR are shown in Figure 4-2. At 90% similarity coefficient, there were five major clusters among the *Salmonella* isolates. Collectively, *S*. Brandenburg isolates were nearly identical as they grouped at 96.7% SC, which is slightly lower than groupings observed with DGGE (97.4% SC). The greatest variation was observed in *S*. Typhimurium and these isolates were subdivided into 6 groups. Overall, the *S.* Typhimurium isolates had the poorest correlation (88.2% SC) among all the serotypes. Two main groups were detected with *S.* Hadar isolates, the first four isolates had 94.8% SC and the second group 96.8% SC however, both groups were different at SC 90.1%. Interestingly, all the *S*. Derby isolates from Plant A with the exception of one were segregated from isolates from Plant B. Only slight variations were noted in both groups and Plant A isolates were 92.5% similar. All the Plant B *S*. Derby, in addition to one Plant A isolate, were related at 94.0% SC. Both groups of isolates produced distinguishable band patterns and were only genetically related at 76.7% SC.

Discussion

The current study evaluated the discriminatory powers of two well-characterized molecular–based genotyping techniques, REP-PCR and DGGE. REP-PCR is commonly compared to PFGE, the gold standard of genotyping (Swaminathan et al., 2001), but only limited studies have been done contrasting REP-PCR and DGGE. A search of Pubmed revealed comparisons between REP-PCR and DGGE analyzing bacteria such as bifidobacteria (Masco et al., 2005) and lactobacillus, but limited research was

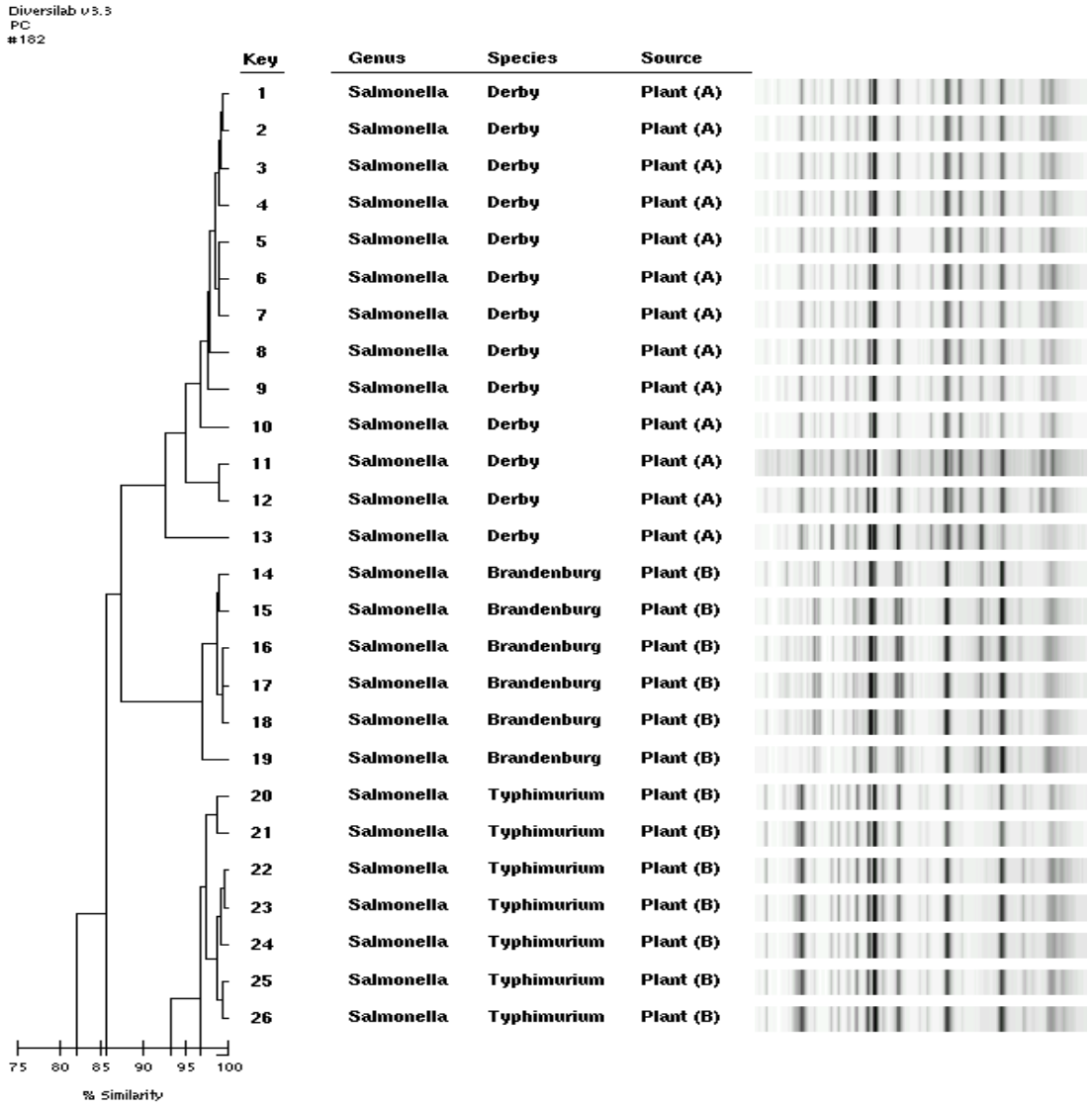

Figure 4-2. Dendrogram shows the repetitive extragenic palindromic-PCR DNA profiles of fifty-four *Salmonella* isolates recovered from two turkey processing plants (A and B). Percentage similarity coefficient is indicated by the bar below the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007).

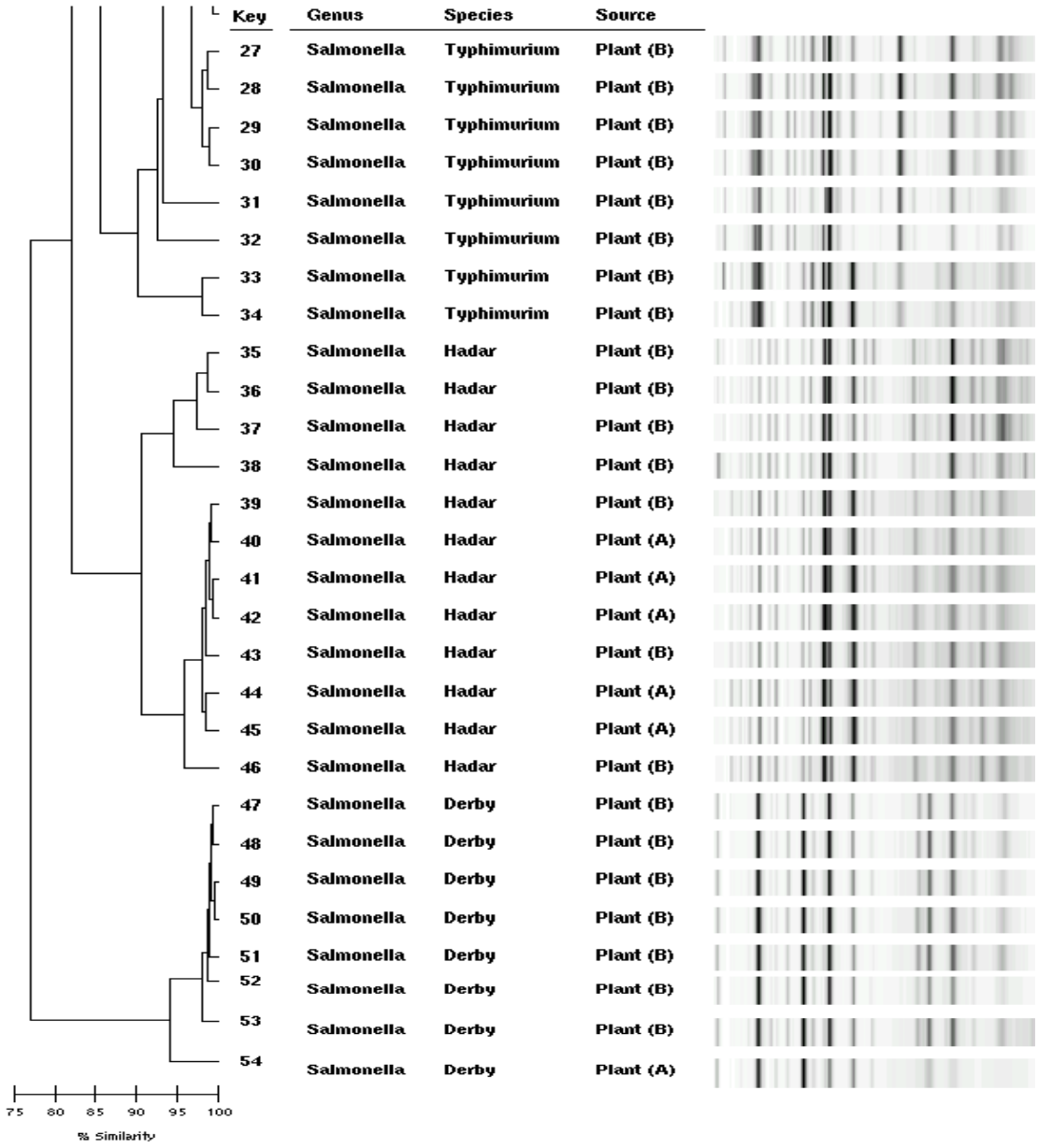

Figure 4-2 Continued

discovered focusing on *Salmonella* or other enteric pathogens. One possible reason could stem from a lack of comparisons between DGGE and REP-PCR could be that many laboratories have already invested in equipment for PFGE (Weigel et al., 2004), and as such may lack the funds to acquire new equipment required for other PCR-based methods of genotyping.

In our investigation, REP-PCR typically generated more band fragments compared to DGGE, thus creating potentially more variability among the fingerprinting profiles. The greater number of bands displayed by REP-PCR compared to DGGE may explain the high degree of variability we observed. Another investigation revealed that REP-PCR is very sensitive and highly discriminatory among bacterial strains (Beyer et al., 1998). Similarly, DGGE displayed sensitivity in foodborne pathogens recovered from several food products (Ercolini, 2004). Both genotypic methods were able to discriminate between the *S.* Derby isolates recovered from the two processing plants sampled in this study, located in two distinct geographical locations in the United States. In addition, the clustering of the *S.* Derby from Plant A with those Plant B isolates (outlier) was differentiated by both fingerprinting techniques. One of the problems often experienced with DGGE is that each gel has to be properly aligned in order to effectively analyze several gels in a comparison. In addition, REP-PCR has limitations, air bubbles in the microfluidics chip can cause dark smears to develop, which may cover DNA bands forcing the sample to be re-evaluated.

Both molecular methods rely heavily on a large data base of *Salmonella* DNA fingerprints for usefulness in identifying unknown isolates. To generate the data base,

the isolates first have to be serotyped, which could be costly, since each *Salmonella* isolate cost US $35 when typed at USDA-APHIS National Veterinary Services Laboratory in Ames, IA. Another alternative to reduce cost is to collaborate with other researchers and acquire isolates that have previously been serotyped.

In our hands, PCR-based DGGE was more economical that REP-PCR in identifying large numbers of *Salmonella* isolates. Without adding the cost of labor, our laboratory calculated the cost per sample using DGGE to be US $12, whereas it cost $27 for a similar evaluation using REP-PCR. One of the most significant advantages to REP-PCR was the reduced time required to analyze a sample. During this study, REP-PCR analyses required 1 h to complete 13 isolates, while the DGGE commonly required 17 hours to run the gel.

Collectively, both techniques were highly discriminatory among *Salmonella* isolates. However, REP-PCR showed a higher variability in the amplicon patterns compared to DGGE, suggesting REP-PCR was able to detect slight variation in the DNA fragments. Both DGGE and REP-PCR displayed high sensitivity in discriminating among *Salmonella* serotypes and either method could be considered as an alternative to more expensive and time consuming, conventional antibody-based serotyping systems.

CHAPTER V

TRACKING OF *SALMONELLA* SEROTYPES FROM THE BROILER FARM TO THE PROCESSING PLANT USING PCR-BASED METHOD

Introduction

Salmonella is one of the predominant bacterial foodborne pathogens affecting humans and it has gained considerable attention both nationally and internationally (Byrd et al., 1999; Fuzihara et al., 2000; Lee et al., 2007). A recent global study conducted by the World Heath Organization estimated that there are approximately 21.6 million cases of typhoidal fever occurring annually (Crump et al., 2004). In contrast, nontyphoidal *Salmonella* is estimated to be responsible for 1.3 billion cases of human salmonellosis and with 3 million deaths annually (Pang et al., 1995). In the United States, nontyphoidal *Salmonella* is responsible for causing 1.4 million illnesses every year, whereas, *Salmonella* Typhi affects 824 persons (Mead et al., 1999). Annually, *Salmonella* has an economic impact of $2.4 billion to the United States economy (USDA-ERS, 2005).

Historically, numerous food products have been implicated as sources or potential sources of *Salmonella* infection in humans, including poultry meat and eggs, pork, fish, milk, spinach, fruits, and vegetables (Molbak et al., 2006). Of these, given the importance of poultry as a worldwide protein source for humans, poultry meat and eggs have been targeted as the primary vehicle for *Salmonella* contamination for human infection (Sarjeant et al., 2005). Consumption of raw or undercooked meats has been

associated with numerous *Salmonella* outbreaks. Eating shell eggs contaminated with *S.* Enteritidis was estimated to affect 182,000 persons (Schroeder et al., 2005). A study conducted in Brazil between 1996 and 2000, concluded that poultry meat was associated with 40% of the *Salmonella* strains isolated from non-human sources (Tavechio et al., 2002).

It has been well established that the different stages in an integrated poultry operation can act as potential environments for *Salmonella* contamination. *Salmonella*-positive eggs from breeder farms can spread the pathogen at grow-out facilities, which could be transferred to processing. Studies have shown that *Salmonella* serotypes isolated from the hatchery and farm were found on processed carcasses (Kim et al., 2007; Lee et al., 2007). Other sources of *Salmonella* contamination include feed, litter, wild birds, rodents, insects, farm workers, and live-haul or transport crates (Bailey et al., 2001; Molbak, 2005). There is still some debate over the importance that feed might play in *Salmonella* contamination. Several researchers have reported that feed is not a major vehicle for *Salmonella* (al-Nakhli et al., 1999; Jones and Richardson, 2004).

Microbial contamination of poultry carcasses at processing is of international importance. All consecutive stages of commercial processing are potential environments for *Salmonella* contamination within the processing facility. Estimates for *Salmonella* contaminations of broiler carcasses have been reported in major poultry producing countries of the world, including Brazil 42% (Fuzihara et al., 2000), Spain 55% (Capita et al., 2003), and the United States 36% (Bailey et al., 2002). These numbers demonstrate that *Salmonella* in a global problem and researchers, government

employees, extension agents, and producers or processors need to work together to find effective solutions.

There is a growing concern that *Salmonella* serotypes detected at the hatchery, feed mill, and grow-out facility persist on the animal and then potentially end up on processed carcasses following processing. With the advent of PCR-based methods for molecular fingerprinting, such as DGGE, it is now possible to track *Salmonella* isolates through an integrated poultry system in epidemiological investigations aimed at identifying successful reduction or intervention strategies. DGGE has been known to be reliable, reproducible, sensitive, and rapid in identifying or characterizing pathogens. DGGE exploits ribosomal DNA (rDNA) fragments of the conserved region that lies next to the hypervariable V3 region of the 16S rDNA gene (Muyzer et al., 1993). DGGE is used to separate nucleic acid fragments that are identical in length, but of different nucleotide sequences (Muyzer et al., 1993; Wawer and Muyzer, 1995). The objective of this study was to determine the genotypic relatedness following DGGE analyses of *Salmonella* isolates recovered throughout an integrated broiler production operation.

Materials and Methods

Sample Collection

The *Salmonella* isolates analyzed in this study were collected from two commercial broiler rearing houses on the same farm and a commercial broiler processing facility in Texas. The two houses were separated from each other by a farm road. Four consecutive broiler flocks from the two houses were sampled to recover the isolates analyzed in this study. Live production samples from both houses were taken on the day

of placement (d 0), and on days 14, 28, and 42 of grow-out. During sampling of Flock 4, scheduling difficulties prohibited data collection from d 0 for this flock. Additionally, in Flock 4 on d 42, *Salmonella* was not recovered from any of the samples collected. During each sampling day of each flock, *Salmonella* recovery was attempted from the following sample types: ceca (C), boot-covers (B), drag swabs (DS), litter (L), feed (F), and water (W), with n = 20, 2, 4, 10, 3, and 3 samples per sample type, respectively.

All the birds (House 1 and 2) from our study were processed as the first flocks of each day of sampling to reduce the potential for later shift contamination. Processing samples consisted of taking 15 samples per each grow-out house at the following locations: live-haul / live-hang carcass rinse (LB), live-haul / live-hang ceca, pre-evisceration carcass rinse (PRE), post-evisceration carcass rinse (PE), and post-chill carcass rinse (PC). Carcass rinsing consisted of adding 400 mL of buffered peptone water (BPW) to a sterile plastic bag and rinsing carcasses by manual shaking or agitation for approximately 1 min. Carcass rinse fluid was then collected aseptically into a sterile plastic culture bottle. All *Salmonella* samples were immediately packed on ice in coolers and transported to the laboratory for culturing.

Salmonella *Culturing*

Twenty-five grams of collected litter were suspended in seventy-five milliliters of BPW and incubated overnight at 37 C. Similarly, 50 g of feed were diluted in 100 mL of BPW. Subsequently, 0.1 mL of overnight litter and feed culture was used to inoculate 10 mL of tetrathionate each. Boot-covers and drag swabs were directly cultured in 130 mL and 75 mL of tetrathionate, respectively. A tenth of a milliliter (0.1 mL) of water

samples were directly cultured in 10 mL of tetrathionate broth. Each cecum was incised longitudinally and directly placed in 10 mL of tetrathionate broth. Live-bird and carcass fluid collected at (PRE, PE, and PC) were incubated at 37 C for 24 h, then 0.1 mL was used to inoculate 10 mL of tetrathionate broth. All samples in tetrathionate broth were incubated at 42 C for 24 h. The next day, 0.1 mL of samples enriched in tetrathionate broth was transferred to 10 mL of Rappaport-Vassiliadis (RV) broth and incubated at 42 C for 24 h. Following RV enrichment, the samples were plated on XLT-4, BGA, and modified lysine iron agar (MLIA) plates and incubated at 37 C for 24 h.

All presumptive *Salmonella* positive isolates were subcultured on TSA and LIA slants for biochemical confirmation of presumptive positives. Subsequently, serological agglutination using polyvalent O and H *Salmonella* antisera was performed on each isolate. All positive *Salmonella* isolates were placed in TSB containing 20% (vol/vol) glycerol and stored at -80 C until needed for DGGE analyses.

DNA Extraction

Approximately 10 µL of *Salmonella* from a frozen stock was streaked onto BGA (containing 25 µg/mL novobiocin) and grown overnight at 37 C for 18-24 h. A colony from each BGA plate was placed in 200 µL of TE (10 mM Tris/1 mM EDTA, pH 8.0) and boiled in a water bath for 15 min. The isolates were chilled then centrifuged at 8000 x *g* for 10 min. The supernatant from each isolate was removed and placed in a clean 200- mL PCR tube. DNA concentrations were standardized to 30 ng/µL (ND-1000, NanoDrop Technologies, Wilmington, DE), then stored at -20 C until needed for DNA amplification.

PCR Amplification

The primers used in the study were modified from Bakshi et al. (2002): forward G_1 5' GAAGTCGTAA-CAAGG-3' and reverse L_1 5'- CAAGGCATCCACCGT-3' (Integrated DNA Technologies). A 30-base GC-rich nucleotide clamp 5'- CGCCCGC-CGCGCGCGGCGGGCGGGGGGGCGGGG-3' was attached to the 5' end of the G_1 primer. The primers (50 pmol of each) were combined with Jump Start Red-Taq Ready Mix (Sigma Chemical Company, St. Louis, MO), 1 µL bovine serum albumin (10 mg/mL), 2 µL of DNA template (30 ng/µL) and deionized water to make a final 50-µL reaction mixture. PCR was performed in a PTC-200 themocycler (MJ Research, Inc., Watertown, MA) as previously described (Hume et al., 2003): 1) initial denaturing at 94.9 C for 2 min; 2) subsequent denaturation at 94 C for 1 min; 3) annealing at 67 C for 45 sec; –0.5 C per cycle touchdown to minimize spurious by-products (Don et al., 1991; Wawer and Muyzer, 1995); 4) extension at 72 C for 2 min; 5) repeat steps 2 to 4 for 17 cycles; 6) denaturation at 94 C for 1 min; 7) annealing at 58 C for 45 sec; 8) repeat steps 6 to 7 for 12 cycles; 9) extension at 72 C for 7 min; and 10) held at 4 C for the final stages.

Gel Electrophoresis

Denaturing gradient gel electrophoresis DNA fragments were resolved on an 8% (vol/vol) polyacrylamide-bisacrylamide gel (37.5:1) with a gradient of 35 to 45% (100% denaturing acrylamide: 7 *M* urea and 40% deionized formamide). Next, 8 µL of PCR product were mixed with an equal volume of 2x loading buffer [0.05% (wt/ vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol, and 70% (vol/vol) glycerol] and 7 µL

was placed in each sample well (20-well comb). Gel electrophoresis was performed using 1x TAE buffer (20 mM Tris, 10 mM sodium acetate, and 0.5 mM EDTA, pH 7.4) in a DCode Universal Mutation Detection System (Bio-Rad laboratories) at 59 C for 17 h at 60 V.

DNA Fingerprint Analysis

All DGGE gel images were digitalized (Alpha Imager®, Alpha Innotech Corporation, San Leandro, CA) and saved in TIFF format. The DNA fragments were analyzed and the dendrogram was generated using the Molecular Analysis Fingerprinting Software, version 1.6 (Bio-Rad Laboratories) and the Dice similarity coefficient and the unweighted pair group method using arithmetic averages (UPGMA) for clustering. The relatedness of each DNA fingerprint and clusters is expressed as a similarity coefficient percentage bar above the dendrogram (Hume et al., 2003). Each *Salmonella* isolate DNA profile was compared against known serotypes to determine genetic relatedness.

Results

In all the flocks, the results for House 1 and 2 were combined on each of the dendrogram. More *Salmonella* isolates were found in House 2 compared to House 1. House 1 was sampled first in all the flocks, however, care was taken to prevent cross-contamination between houses.

Flock 1

Eighteen *Salmonella* isolates that were recovered from Flock 1 of an integrated broiler operation and subjected to PCR-based DGGE are displayed in Figure 5-1.

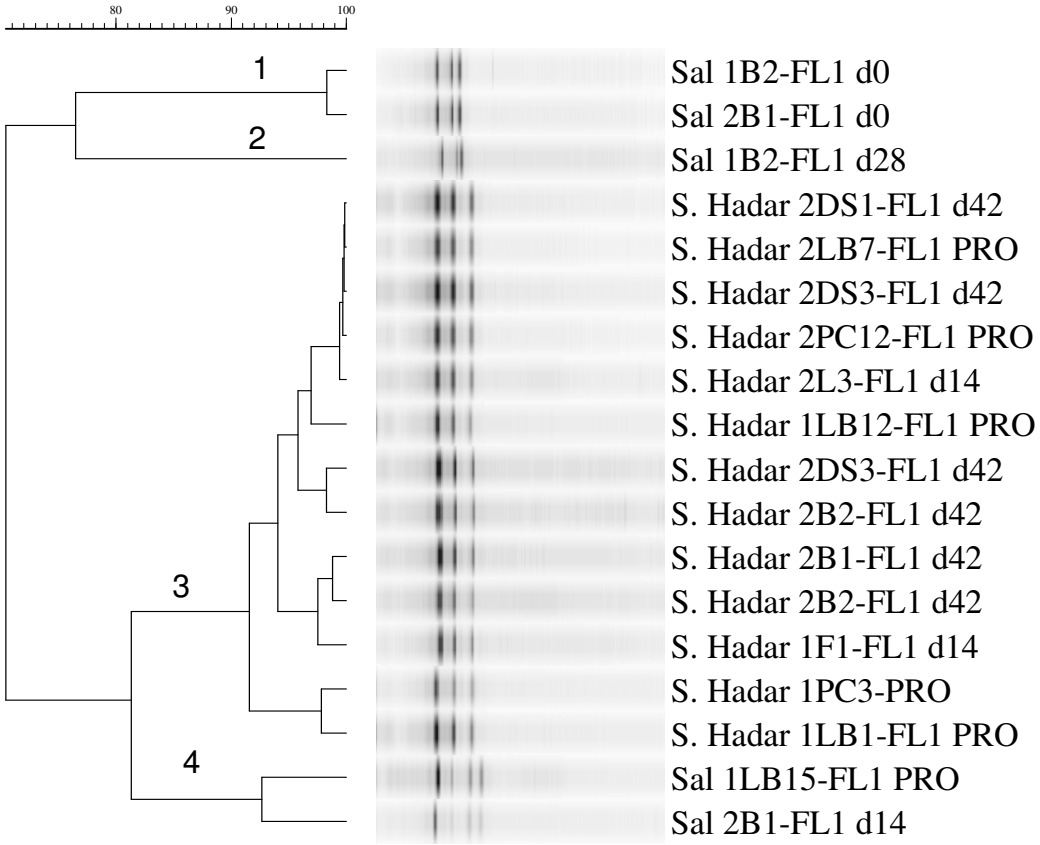

Figure 5-1. Dendrogram of flock 1 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation. Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007). Numbers (1-4) are clusters identification. Key: Sal 1B1-FL1 d 0; *Salmonella* (Sal), House 1, boot-cover # 1 (B 1), flock 1 (FL 1), day 0 (d 0), ceca (C), drag swabs (DS), feed (F), live-haul / live-hang (LB), pre-evisceration (PRE), post-evisceration (PE), post-chill (PC), processing (PRO).

The cut off point of similarity coefficient percentage was set at 90% and 4 clusters were identified. The DNA fingerprints from the two boot-covers obtained at d 0 were identical (Cluster 1), however, were segregated at 76.5% SC from the d 28 boot-cover isolate (Cluster 2). Thirteen of the *Salmonella* isolates grouped in Cluster 3 were identified as *S*. Hadar and were from all sampling periods except, d 0 and d 28. Most of the *Salmonella* isolates evaluated in this study were recovered from House 2. The DNA profiles in Cluster 3 show that there is some relationship between *Salmonella* recovered from the farm on d 14 and at processing. All thirteen DNA patterns were hosted at 91.5% SC. The live-bird (House 1) and boot-cover (House 2) isolates found in Cluster 4 were similar at 92.7% SC, however, were separated from the remaining isolates at 81.3% SC.

Flock 2

A total of 31 samples during Flock 2 sampling were *Salmonella*-positive, with 11 and 20 isolated from House 1 and 2, respectively. Based on the DNA pattern analysis, the isolates were segregated into seven groups (Figure 5-2). The first 4 clusters contain 5 samples and showed the highest variation among all clusters. The drag swabs and boot-cover isolates obtained from House 1 on d 14 were similar at 90.6%, but were slightly different from DS at day 28 (Cluster 5). The sixth cluster contains eight isolates and the DNA profiles were identified by genotype profile comparison to known serotypes as *S*. Hadar. The *S*. Hadar cluster includes *Salmonella* profiles from litter and drag-swab on d 14 (House 1), d 42, and processing (House 2). There was little variation in amplicon pattern identified within the group.

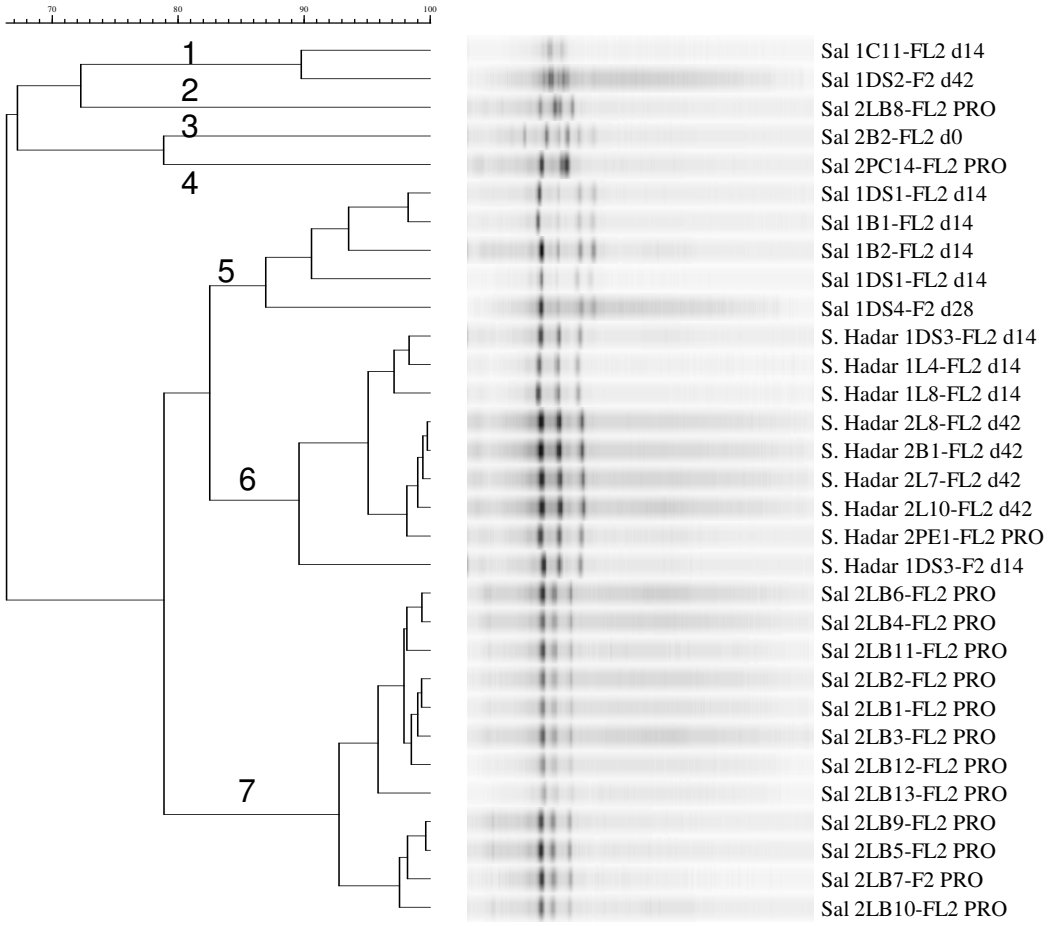

Figure 5-2. Dendrogram of flock 2 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation. Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007). Numbers (1-7) cluster identification. Key: 1C11-FL2 d 14; House 1, ceca # 11 (C 11), flock 2 (FL 2), day 14 (d 14), boot-covers (B), drag swabs (DS), feed (F), live-haul / live-hang (LB), pre-evisceration (PRE), post-evisceration (PE), post-chill (PC), processing (PRO).

There was a distinct separation of House 1 and 2 fingerprints, except for one drag swab isolate. *Salmonella* detected on d 14 (House 1) were very related (95.1% SC) to litter, boot-cover, and post- evisceration (House 2) obtained at d 42 and processing. The outlier was shown to be distinct (89.6% SC) from the others. The final cluster contained 12 live-bird samples from House 2 and grouped at 92.8% SC.

Flock 3

The largest number (n=78) of *Salmonella*-positive samples were identified in Flock 3 and four distinct DNA profiles were observed. The dendrogram was divided into three sections for viewing due to the large number of isolates which were compared from this flock. Based on a threshold of 90% correlation, the band patterns were divided into four groups: 1, 2, 3, 4, with n= 29, 28, 3, and 18, respectively (Figure 5-3). In Cluster 1, *Salmonella* frequency was lowest in water and feed. The water profile differ slightly (97.7% SC) from live-bird, cecal, and post-chill. Boot-cover, cecal, and litter isolates recovered at d 14 from House 1 showed a degree of similarity to samples obtained from House 2 on d 14 and 28. Also, there was only a slight variation in band fragment pattern between feed, litter, and cecal or live-haul/live-hang isolates analyzed on d 42 and processing. Collectively, Cluster 1 exhibited a 91.6 % similarity coefficient. The second cluster with 26 isolates was similar to *S*. Hadar and contained isolates from all sampling days except of d 14. Feed and drag swab collected on d 0 were of the same genotype (Cluster 3). *Salmonella* fingerprints recovered on d 42 were comparable to some isolates observed throughout the processing facility. Interestingly, pre-eviscerated carcasses were segregated from the other members of the group at 93.5 % similarity.

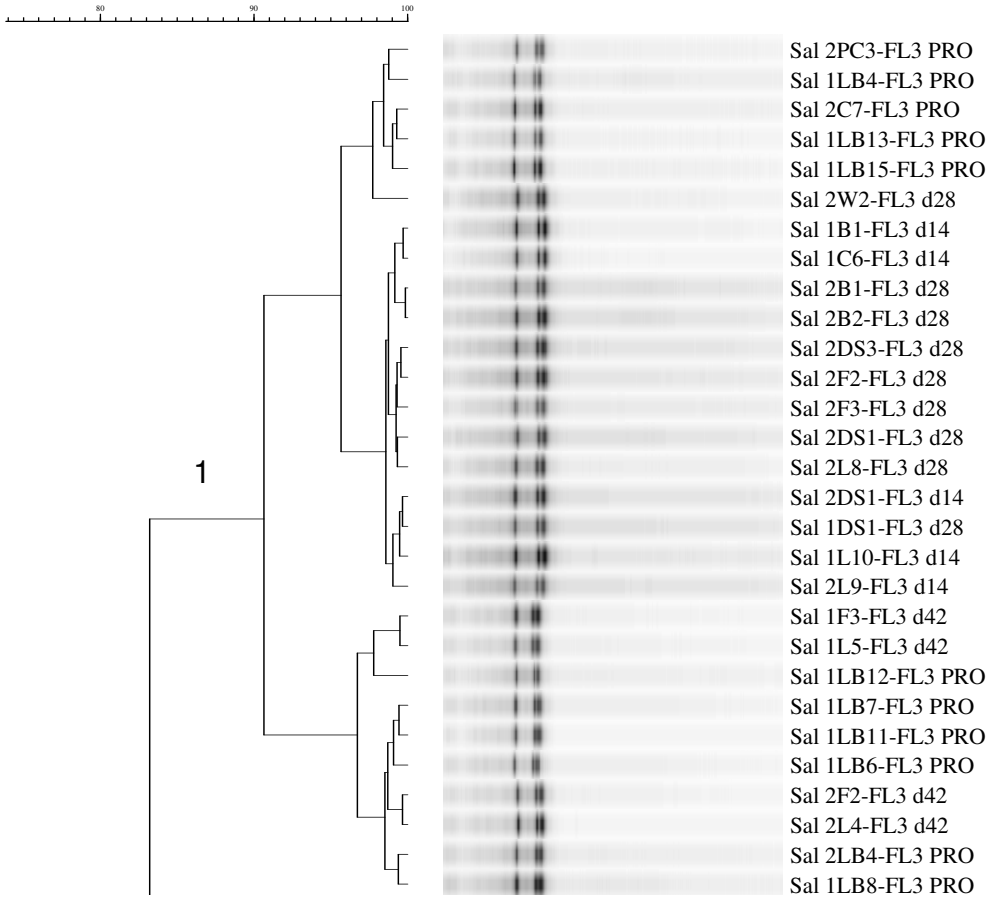

Figure 5-3. Dendrogram of flock 3 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation. Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007). Numbers (1-4) cluster identification. Key: 2W2-FL3 d 28; House 2, water # 2 (W 2), flock 3 (FL 3), day 28 (d 28), ceca (C), boot-covers (B), drag swabs (DS), feed (F), live-haul / live-hang (LB), pre-evisceration (PRE), post-evisceration (PE), post-chill (PC), processing (PRO).

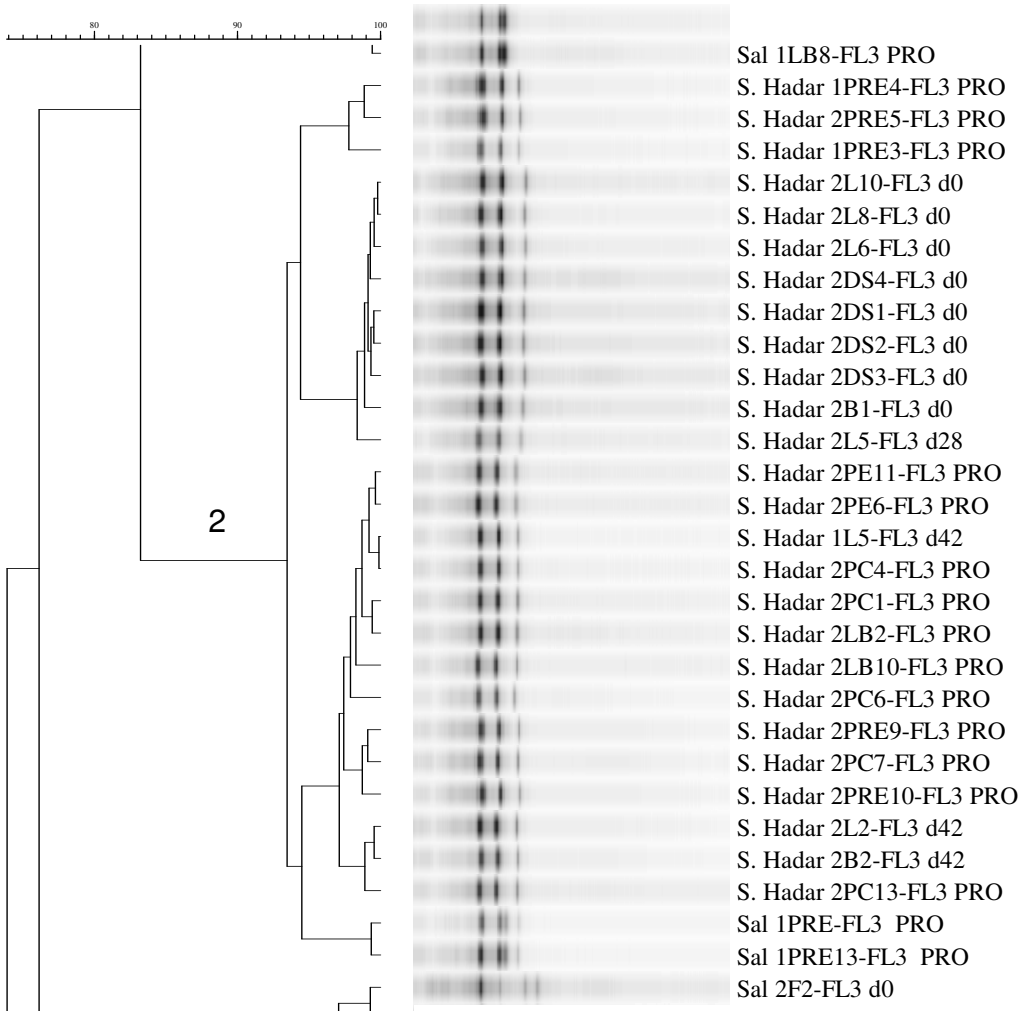

Figure 5-3 Continued

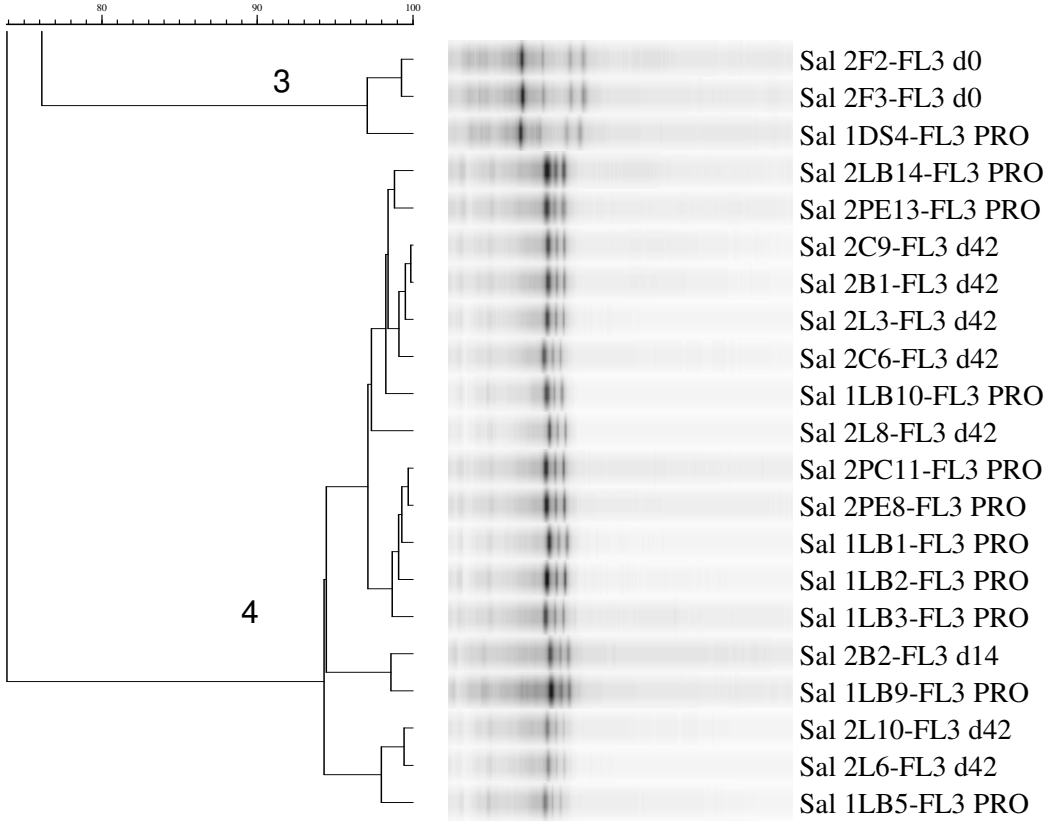

Figure 5-3 Continued

Salmonella present on drag swabs and feed identified on d 0, exhibited 97.1% similarity. The forth cluster demonstrated close association (94.3% SC) between samples collected on d 14, 42, and processing.

Flock 4

Twenty-nine *Salmonella* isolates were collected in Flock 4 and the dendrogram is divided into four main clusters (Figure 5-4). Due to scheduling conflicts, no data was obtained on d 0. Additionally, none of the samples collected on d 42 were found to be *Salmonella*-positive. The DNA prolife of the second group was genetically related to *S.* Hadar and contains only isolates from live bird wash at processing. Overall, the eight samples showed 95.4% SC among the group. The first and second clusters were different at 77.5%. Cluster 3, had the highest variation among the groups and was subdivided into three units. The post-chill sample recovered from House 1 was only separated from other processing samples at 96.7% similarity. The comparison of the litter collected on d 14 with processing isolates yielded a 90.8% correlation. The two amplicon patterns from the pre-evisceration carcass rinse positive samples were the same, but were 87.5% unrelated to litter and boot-cover positive *Salmonella*. There was a very close correlation (97.7%) between litter and boot-covers obtained on d 14 and 28 in both houses. All twenty-nine *Salmonella*-positive amplicons shared at 69.2% similarity.

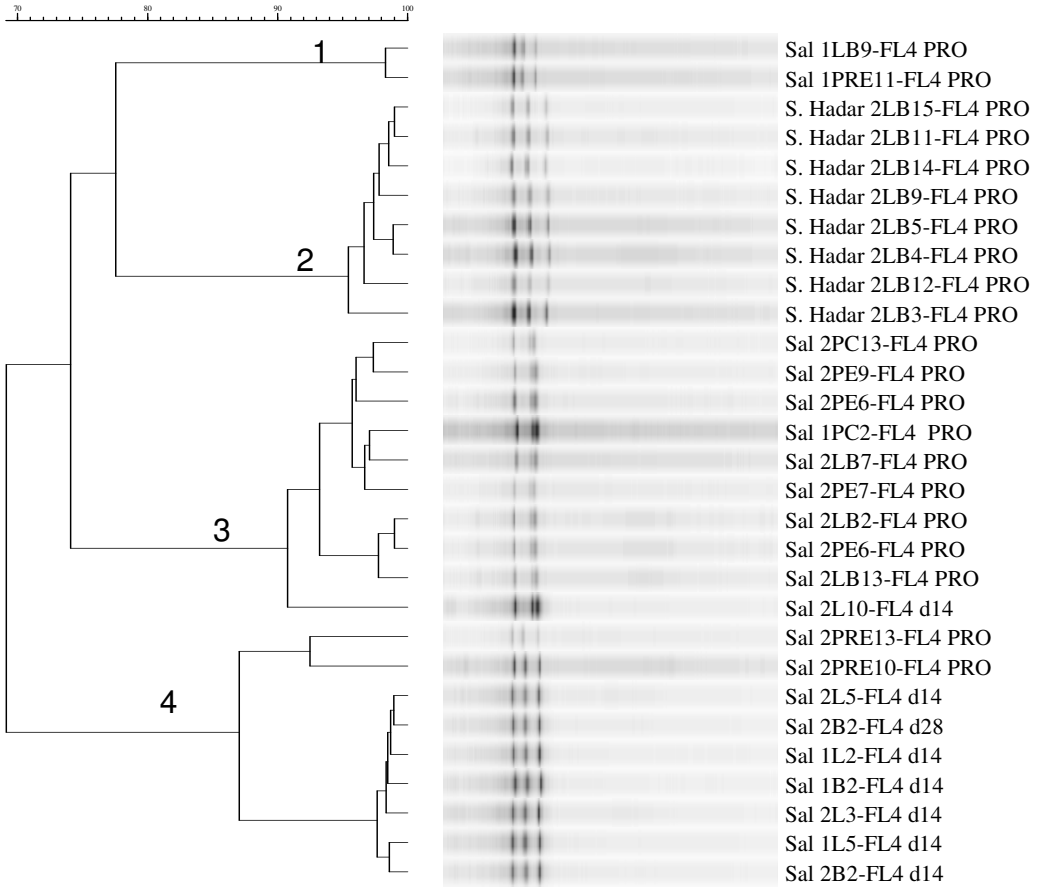

Figure 5-4. Dendrogram of flock 4 denaturing gradient gel electrophoresis band patterns (16-23S rDNA) of *Salmonella* isolates from an integrated poultry operation. Percentage similarity coefficient is indicated by the bar above the dendrogram; ≥ 92% are very related or the same, 85 to 91% are similar, 80 to 84% are somewhat similar, and ≤ 79% are unrelated (Dunkley et al., 2007). Numbers (1-4) cluster identification. Key: 2L5-FL4 d 14; House 2, litter #5 (L5), flock (FL), day 14 (d 14), ceca (C), boot-covers (B), drag swabs (DS), feed (F), water (W), live-haul / live-hang (LB), pre-evisceration (PRE), post-evisceration (PE), post-chill (PC), processing (PRO).

Discussion

Several researchers have reported on the tracking of *Salmonella* serotypes throughout an integrated poultry operation (Bailey et al., 2001; Kim et al., 2007; Lee et al., 2007; Tavechio et al., 2002). Most of these reports focused on *Salmonella* tracking using conventional techniques, but recently other reports have surfaced which have begun exploring PCR-based methods (McCrea et al., 2005; Nde et al., 2006; Olah et al., 2005). To date, only limited studies have been undertaken that utilized PCR-based techniques such as DGGE to generate DNA fingerprints of *Salmonella*. DGGE has long been utilized in gut ecology studies and recently the procedure has emerged as a new technique in epidemiological investigations targeting foodborne pathogens.

The current study focused on characterizing *Salmonella* recovered from four flocks of broilers from placement in grow-out houses through commercial processing. The stage of the poultry production and processing that is most likely to result in the greatest potential for *Salmonella* transfer to the final product continues to be a subject of debate among researchers and health care professionals. Our conclusion suggests that *Salmonella* identified on carcasses at processing were more likely to trace back to the *Salmonella* the animal was exposed to during live-production as compared to the hatchery (Bailey et al., 2001). An alternative view, derived from a Belgian study, found no correlation between *Salmonella* serotypes detected at processing with those detected during grow-out (Heyndrickx et al., 2002). The current study demonstrates that there was correlation between *Salmonella* fingerprints recovered at pre-harvest and post-harvest sampling locations.

During the first flock sampled, only limited numbers of *Salmonella*-positive isolates were recovered. Three different fingerprint patterns were identified, suggesting that there were three predominant *Salmonella* serotypes present in the flocks. However, only one-cluster fingerprint profile shared similarity to the *S*. Hadar in the DNA-bank. *S*. Hadar is a common serotype of poultry, supported by Nde et al. (2006) showing that 71% of the *Salmonella* serotypes isolates at a turkey processing plant were *S*. Hadar. While a recent federal survey of serotypes isolated during broiler processing identifies Kentucky, Enteritidis, Heidelberg and Typhimurium as the predominant isolates recovered, as recently as 2001, *S*. Hadar was considered as one of the top broiler serotypes (USDA-FSIS, 2007).

S. Hadar isolates recovered in the live production environment were also present on carcasses at processing. To effectively compare unknown isolates against the data base, it is important to have a large data base of *Salmonella* serotypes. Our data bank contained 70 isolates, but only 15 different serotypes are present. Therefore, representative isolates from each cluster will be sent for serotyping. The *Salmonella* isolates detected at day of placement were not related to any of the other isolates recovered while sampling this flock. One of the boot-cover isolates and a live-haul / live hang rinse isolate showed a 92.7% similarity, however, both samples were from different houses. Since these patterns were not seen elsewhere within this flock, there is a possibility that the pathogen was transferred between the houses.

Similar to Flock 1, *S*. Hadar was detected in Flock 2 and none of the other DNA profiles matches with the known serotypes in our limited data base. The high variation in Cluster 7 was due to different shade intensities of the fragments. *Salmonella* Hadar was more prevalent in House 1 at d 14, whereas in House 2, only d 42 and a PE were positive. It appears that the litter was heavily contaminated with *Salmonella* serotypes including *S*. Hadar and should be thought of as a carrier between flocks. With the exception of the first four profiles that were not detected elsewhere, all remaining patterns were similar to those from other flocks. The LB isolates recovered at processing were not related to any of the d 42 litter samples, suggesting that the birds may have been colonized / infected during transportation or that older birds became more resistant to the *Salmonella*.

The *Salmonella* isolates identified in Flock 3 were separated into four clusters, suggesting that the isolates belong to four different serotypes. With seventy-eight entries in Flock 3, more correlation between pre-harvest and post harvest was noted. In the third cluster, fingerprint patterns from d 0 feed and a drag swab were detected, this pattern only showed association with Flock 1. The reason for this serotype not being recovered after d 0 could be related to feed changes. In addition, changes in nutrients level and texture of feed could influence *Salmonella* recovery. Interesting, the DNA fingerprints of the last cluster were detected only at d 42 and processing. Therefore, this unfamiliar serotype could have been introduced from others houses not participating in the study. *Salmonella*-positive water, and cecal samples were rarely detected in the study, however

samples of this type were positive in Flock 3. The final cluster exhibited similar patterns between the two houses.

Flock 4 revealed four types of DNA profiles depicting four distinct serotypes being predominant. Although the study was done in winter, only twenty-nine *Salmonella*-positive isolates were detected. High levels of *Salmonella* recovery are expected in the winter months compared to other seasons. Bailey et al. (2001) reported that *Salmonella* recovery at pre-harvest is highest in the winter and lowest in the summer months. The low total number of *Salmonella* isolates evaluated in this flock can be attributed to lower sampling frequency, as samples were not taken on day 0 due to a scheduling conflict. Additionally, none of the d 42 isolates collected resulted in a confirmed *Salmonella* positive sample. As such, without any d 42 isolates for comparison, it is more difficult to determine if any of the processing fingerprints originated from the farm or if the birds were contaminated during transportation. Due to the low number of isolates recovered in this flock, especially in House 1, there was not enough data to correlate pre- and post-harvest profiles.

The observations of this investigation revealed that PCR-based DGGE created distinct fingerprints that represent different *Salmonella* serotypes. One limitation that we experienced during our analyses was linked to data base size from which *Salmonella* serotype estimates can be derived. To effectively compare DNA fingerprints of unknown isolates with the data base, it is important to generate a large library, which increases the probability of finding a match between the unknown isolate and an established genotype of a specific *Salmonella* serotype. With the limited number of different serotypes in our

data base (Tables 3-1 and 3-2), only *S.* Hadar fingerprints were related to some of the unknown isolates. Although it may be labor intensive and expensive to create the data base, this will allow for fast identification of *Salmonella* isolates. Typically, it cost US $35 and can take up to 4 wk to serotype a *Salmonella* isolate. In our study, DNA extraction was performed by boiling the cell, which is a relatively cheap process considering no extraction kits were used. Although the gel was ran for 17 h, most of the time is spent running the gel and only little time is required for set up. During a *Salmonella* outbreak, a PCR-based typing method such as DGGE which is rapid, sensitive, and reproducible could prove to be very useful for epidemiological and diagnostic purposes.

Numerous primers are available within the literature which allow researchers to amplify the hypervariable V3 region between the 16S and 23S rDNA, making the technique easy to perform. The attachment of GC-rich nucleotide clamp to the 5' end of one of the primer will prevent the DNA from totally denaturing until it reaches the lowest meting domain. DGGE like other PCR-based techniques have some drawbacks. DGGE only separates PCR fragments that are less than 500 base pairs in length (Muyzer et al., 1998; Roelfsema and Peters, 2005). In addition, DNA fragments with different sequences could be problematic at times to separate due to co-migration of these fragments (Muyzer et al., 1998). According to Ercolini (2004), only a limited number of DNA fragments can be separated due to poor gel resolution. Similarly, DGGE has low sensitivity to microbes that are present in limited quantities (Muyzer, 1999).

We feel the current study demonstrates that there is correlation between *Salmonella* isolates recovered at pre- and post-harvest based upon fingerprinting profiles generated by DGGE genotyping. Overall, eight different DNA profiles were presented when all the data was pooled, suggesting that eight serotypes were isolated during the course of our study (dendrogram not shown). With the exception of one serotype found in Flock 3, all the others were identified in all the flocks. On the farm, litter was the most contaminated sample type within the live-production environment and may have been the major reservoir for *Salmonella* between the consecutive flocks sampled during this study.

CHAPTER VI

CONCLUSIONS

Salmonella is regarded as the major bacterial foodborne pathogen causing human illnesses worldwide. It cannot be debated that poultry meat and eggs are major vehicles for *Salmonella* transmission to human. As a food safety issue, the regulatory bodies have imposed performance standards to which federally inspected processing plants must comply. As such, researchers, veterinarians, processers and the government are working in tandem to search for new method of testing and characterization for *Salmonella*. PCR-based methods for identifying pathogens provide more advantageous options for this purpose than conventional testing.

Experiment 1 reveals that *Salmonella* serotypes isolated during commercial turkey processing may vary dependent upon geographical (plant) location and within each plant, by sampling site. Furthermore, results from the current study showed that *Salmonella* isolates of the same serotypes, but from different geographical locations in the United States may differ in band profile. Six different serotypes were isolated from Plant A, whereas in Plant B ten serotypes were identified. The two primary *Salmonella* serotypes recovered from the turkey processing plant were *S*. Derby (Plant A) and *S*. Typhimurium (Plant B). All the *Salmonella* isolates were evaluated using PAGE and DGGE. It was observed that DGGE was more sensitive than PAGE in detecting and characterizing *Salmonella*. Creating a library or genotypic data base of *Salmonella*

serotypes would allow for rapid identification of unknown serotypes and could function as a preemptive tool until more conventional definitive serotypic identification is made.

In the second experiment, four *Salmonella* serotypes from the previous study: Brandenburg, Derby, Hadar, and Typhimurium were subjected to DGGE and REP-PCR to determine the discriminatory powers of each procedure for pathogen identification. The study highlighted that REP-PCR generated more fragments per isolate, and was able to detect small differences in amplicon profile. Therefore, REP-PCR could be considered more discriminatory than DGGE. Although automated REP-PCR was able to provide DNA fingerprints in a relatively shorter period of time than DGGE, it should not be over looked that DGGE is more economical than REP-PCR in analyzing large number of samples. Collectively, both techniques were able to differential differences in band patterns of *S*. Derby from the two different locations.

In Experiment 3, *Salmonella* serotypes were tracked in a commercial integrated broiler complex to the processing plant. The different stages of the poultry operation may become potential environment for carcass contamination. Some isolate fingerprints detected at grow-out were similar to patterns at processing. Only *S*. Hadar from our data base was related to unknown isolates. It was also observed that flocks with fewer isolates showed less correlation between pre-harvest and post-harvest serotypes. One limitation that we experienced during our analyses was linked to data base size from which *Salmonella* serotype estimates can be derived. Therefore, to adequately use molecular-based techniques to identify *Salmonella* it is important to generate a large library, which increases the probability of finding a match.

Our studies demonstrate that molecular techniques could be considered as an alternative to serotyping. PCR-based methods are relatively cheap compared to convention methods, however, a large data base is needed for PCR-based methods.

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