ABSTRACT

KEELARA VEERAPPA, SHIVARAMU. Molecular Epidemiology of *Salmonella* Isolated from Pigs Reared in Distinct Swine Production Systems and Humans. (Under the direction of Dr. Siddhartha Thakur).

Salmonella is one of the most important foodborne pathogens causing salmonellosis in both humans and animals. Emergence and persistence of antimicrobial resistant (AMR) foodborne pathogens due to routine use of antimicrobials for therapeutic, preventive and growth purposes in conventional food animal production and its association with the development of AMR bacterial strains is a growing public health concern. Therefore, increasing consumer preference of antimicrobial-free (ABF) meat products makes it important to identify risk factors associated with the transmission of AMR foodborne pathogens in these unique production systems.

The objectives of this study were to 1) determine the dynamics of *Salmonella* in pigs reared in distinct production systems, 2) characterize and compare isolates from pigs, the environment and humans at the phenotypic and genotypic level, and 3) identify conjugative plasmids and determine their inter- serovar exchange among *Salmonella* of human, pig and environmental origin. To address the above objectives, we collected pig and environmental samples from 10 conventional and eight ABF longitudinal cohorts at different stages of production and slaughter. A total of 1,090 *Salmonella* isolates was recovered with a significantly higher prevalence in conventionally reared pigs (4%) and their environment (12%) compared to ABF pigs (0.2%) and their environment (0.6%) (P<0.001). There were 24 different serotypes identified with *Salmonella* Typhimurium, *Salmonella* Anatum,

Salmonella Infantis, *Salmonella* Rissen and *Salmonella* Derby being predominant. At the same period of time we collected 572 human clinical *Salmonella* isolates from North Carolina state laboratory of public health.

Antimicrobial susceptibility testing revealed that conventional isolates exhibited higher AMR than human clinical and ABF isolates. Overall, the highest frequency of resistance in *Salmonella* isolates was against TET, followed by FIS and STR with the exception of AMI and CIP resistance which were only exhibited by human clinical isolates. We also detected resistance to 3rd and 4th generation of cephalosporins by *Salmonella* isolates of three different sources. Conventional isolates also had a higher frequency of multidrug resistant (MDR) isolates compared to human and ABF isolates. We detected multiple AMR genes including bla_{CMY-2}, bla_{TEM}, bla_{PSE}, *cml*A, *strA/B*, *aadA1/A2, aph*AI, *tet(A),(B),(C)* and *(G)* along with class I and II integrons among *Salmonella* isolates.

Genotyping of temporally and spatially related *Salmonella* isolates from human clinical cases, pigs and their environment from two production systems by Pulsed field gel electrophoresis (PFGE) analysis revealed a similar fingerprint profile among the *S*. Typhimurium serotype, which is commonly associated foodborne outbreaks, among all three sources. Detection of 100% genotypic similarity among serotypes isolated from pigs and their environment belonging to the same cohort at different stages of production at farm and slaughter potentially explains the dissemination of *Salmonella* throughout the production chain.

The potential development and propagation of MDR in *Salmonella* through mobile genetic elements was further evidenced by identification of conjugal plasmids in MDR

Salmonella isolates. Characterization of these plasmids with restriction enzymes and southern hybridization provided clear evidence of the presence of similar plasmids circulating among *MDR Salmonella* isolates of human, pig and environmental origin.

In conclusion, our study results demonstrate the presence of AMR *Salmonella* in ABF and conventional production systems at farm, slaughter and the environment. The phenotypic and genotypic profiles underscore the potential role played by the environment in the persistence and dissemination of AMR *Salmonella* in the two production systems and in humans. Dissemination of identical conjugal plasmids carrying multiple resistance determinants and its inter-serovar irrespective of source of origin is essential to understand the spread and persistence of antimicrobial resistance in the food chain. Further studies needed to determine the role played by the environment in occurrence and dissemination of AMR *Salmonella* in the food chain.

Molecular Epidemiology of *Salmonella* Isolated from Pigs Reared in Distinct Swine Production Systems and Humans

by
Shivaramu Keelara Veerappa

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2013

APPROVED BY:

Siddhartha Thakur
Committee Chair

Paul Orndroff
Committee member

Glen Almond
Committee member

Jonathan Olson
Committee member

DEDICATION

To My Grandparents

Chikkenamma and Eregowda,

To My Parents

Veerappa and Jayamma,

To My Sisters, Brothers and Friends

for their endless love, encouragement, support, and sacrifices

BIOGRAPHY

Shivaramu Keelara Veerappa was born November 22nd, 1982 in Keelara, Karnataka, India. He attended the Bengaluru Veterinary College, Karanataka Veterinary Animal and Fishery University, Bidar where he graduated with Bachelors of Veterinary Science and Animal Husbandry in 2005. He was awarded a Junior Research Fellowship in 2006 by Indian Council for Agricultural Research to pursue Master's in Veterinary Public Health at Indian Veterinary Research Institute (Deemed University), Izatnagar , UP, from 2006-2008. Then in 2009 he joined the Comparative Biomedical Sciences program at North Carolina State University College of Veterinary Medicine. His Ph.D. work focused on the molecular epidemiology of *Salmonella* in swine production systems and humans, under the direction of Dr. Siddhartha Thakur. He will further his career as a postdoctoral research fellow at Environmental Microbial and Food Safety Laboratory, USDA-ARS.

ACKNOWLEDGMENTS

Happiness lies in pursuit as much as in reaching the goal and today I stand with the accomplishments of my endeavor. While pursuing it, many a known and unknown hands and learned souls put me on the right path and enlightened me with their knowledge and experience. No words could adequately express my feelings; I shall ever remain thankfully indebted to them all.

Indeed the words at my command are just not sufficient to express my sincere thanks and deep sense of gratitude to my advisor Dr. Siddhartha Thakur for his guidance, support, constant appreciation and encouragement. During my entire course of study he has taken a keen interest in me and extended unreserved, unconditional help at all times. I would also like to thank my committee members Dr. Paul Orndroff, Dr. Glen Almond, Dr. Jonathan Olson for agreeing to be part of my committee, for their support and advice.

I would like to extend my special and sincere thanks to my laboratory friends, Macarena, Emma, Patty, Mitsu, Deena, Michelle and Dr. Luke for their wonderful support, company, laughs and unconditional love during the course of study. I would like to thank all of my friends from India, US and overseas for their continuous rejuvenating, inspiring emails, special care and love, encouragement to carry out my program. I will always be indebted to them.

This journey could not have possible without support and love from my family, my parents, aunt and uncle, sisters, brothers, my little niece and nephews for their love, affection and blessings without which this dream could not have been achieved. Last but not the least I am grateful to pigs who shared their samples for my selfish ends.

TABLE OF CONTENTS

LIST OF TABLES .... **x**

LIST OF FIGURES .... **xiii**

1. CHAPTER I: Literature Review **1**

1.1 Introduction 1

1.2 *Salmonella* History, Morphology and Nomenclature 2

1.3 *Salmonella* in Humans, Pigs and Their Environment 4

1.4 Antimicrobial Use in Food Animals and Humans 6

1.5 Antimicrobial Use in Swine Industry and its Impact 7

1.6 Antimicrobial Resistant *Salmonella* and its Public Health Concerns 9

1.7 Antimicrobial Resistance Mechanisms 11

References 14

2. CHAPTER II: Longitudinal study of distribution of similar antimicrobial resistant *Salmonella* serovars in pigs and their environment in two distinct swine production systems **38**

2.1 Abstract 38

2.2 Introduction 39

2.3 Materials and methods 41

2.3.1 Study design and sample source 41

2.3.2 Sampling on farm 43

2.3.3 Sampling at slaughter facility 44

2.3.4 *Salmonella* isolation and confirmation 45

2.3.5 *Salmonella* serotyping 46

2.3.6 Antimicrobial susceptibility testing 46

2.3.7 Pulsed Field Gel Electrophoresis (PFGE) analysis 47

2.3.8 Statistical Analysis 48

2.4 Results 49

2.4.1 *Salmonella* prevalence in pigs and the environment at farms 49

2.4.2 *Salmonella* prevalence in carcasses and the environment at slaughter 51

2.4.3 Identification and distribution of *Salmonella* serotypes 51

2.4.4 Antimicrobial Resistance profile of *Salmonella* 52

2.4.5 Distribution and association of MDR patterns with *Salmonella* serotypes 53

2.4.6 Pulsed Field Gel Electrophoresis (PFGE) 54

2.5 Discussion 56

2.6 Conclusions 62

2.7 Acknowledgements 63

2.8 References 64

3. CHAPTER III: Comparative phenotypic and genotypic characterization of temporally and spatially related non-typhoidal *Salmonella* isolated from human clinical cases, pigs and the environment. 95

3.1 Abstract 95

3.2 Introduction 97

3.3 Materials and methods 98

3.3.1 *Salmonella* isolate sources 98

3.3.2 Antimicrobial susceptibility testing (AST) 99
3.3.3 Detection of resistance genes and integrons 100
3.3.4 Pulsed field gel electrophoresis (PFGE) analysis 101
3.3.5 Statistical analysis 101
3.4 Results 101
3.4.1 Antimicrobial resistance profiles 101
3.4.2 MDR patterns identified 102
3.4.3 Molecular characterization of antimicrobial resistance (AR) determinants 103
3.4.4 Pulsed Field Gel Electrophoresis (PFGE) 104
3.5 Discussion 105
3.6 Conclusion 109
3.7 Acknowledgments 110
3.8 Disclosure Statement 110
3.9 References 111
4. CHAPTER IV: Dissemination of identical trans-conjugal plasmids from antimicrobial resistant *Salmonella* serotypes originating from humans, pigs and their environment 134
4.1 Abstract 134
4.2 Introduction 135
4.3 Materials and methods 137
4.3.1 Origin of *Salmonella* isolates 137
4.3.2 Plasmid isolation and its characterization 138

4.3.3 Conjugation experiment, selection and confirmation of trans-conjugants 139

4.3.4 Multiplex PCR for plasmid replicon typing.. 140

4.3.5 Southern hybridization.. 141

4.4 Results.. 142

4.4.1 Plasmid isolation and its characterization.. 142

4.4.2 Conjugation experiment, selection and confirmation of trans-conjugants 143

4.4.3 Multiplex PCR for plasmid replicon typing.. 144

4.4.5 Southern hybridization.. 145

4.5 Discussion.. 145

4.6 References.. 149

5. APPENDICES 162

Appendix 5. 1 Dendrogram exhibiting genotypic similarity among *Salmonella* isolates from ABF production system at farm and slaughter... 163

Appendix 5. 2 Overall *Salmonella* prevalence in ABF and conventional production systems... 166

Appendix 5. 3 *Salmonella* prevalence in ABF and Conventional farms by production stage and sample type.. 167

Appendix 5. 4 *Salmonella* prevalence in ABF and conventional carcass and the environment at slaughter.. 169

Appendix 5. 5 Minimum inhibitory concentration (MIC) levels and frequency of resistance of *Salmonella* isolates from ABF and conventional production systems 170

Appendix 5. 6 Geographical distribution of *Salmonella* isolates from humans, pigs and the environment 174

Appendix 5. 7 Circular map of plasmid I1-Iγ (103kb) isolated from *Salmonella* serovar Anatum of pig origin (S732/pS02) containing placement of 35 genes annotated based on BlastX matches 176

Appendix 5. 8 Circular map of plasmid (91kb) isolated from *Salmonella* serovar Rissen of environment origin (S1004/pS17) containing placement of 35 genes annotated based on BlastX matches 177

LIST OF TABLES

Table 1.1 Number of *Salmonella* serovars per species and subspecies……………………......27

Table 1.2 The top 10 most frequently reported *Salmonella* serovars from humans, 2011.....28

Table 1.3 The top 10 most frequently reported *Salmonella* serovars from pigs, 2011……..29

Table 1.4 Annual sales for antimicrobials used in humans and food animals, 2011……….30

Table 1.5 WHO categories of antimicrobials according to their importance in human medicine……………………………………………………………………………...31

Table 1.6 FDA approved antimicrobials for treatment and growth promotion in swine……34

Table 2.1 Breakdown of *Salmonella* isolation by farm and farm type……………………......72

Table 2.2 Breakdown of *Salmonella* isolation by production/processing stage from ABF and conventional production system...74

Table 2.3 The distribution of *Salmonella* serotypes from pigs and the environmental samples at farm and slaughter (n=1,090)……………………………………………………......75

Table 2.4 The MIC distribution (squashtogram) of *Salmonella* isolated from all swine samples (n=1,090)*……………………………………………………………………..76

Table 2.5 Comparison of resistance and MIC distribution (squashtogram) for *Salmonella* isolated from the conventional production system at farm and slaughter (pigs n=386; environment n=536)*…………………………………………………………….......79

Table 2.6 Distribution of *Salmonella* serotypes associated with predominant MDR patterns in conventional production system…………………………...81

Table 3.1 Geographical distribution of *Salmonella* isolates from humans, pigs and the environment………………………………………………………………………......117

Table 3.2 Minimum inhibitory concentration (MIC) levels and frequency of resistance of *Salmonella* isolates from humans, pigs and environment…………………………………..119

Table 3.3 Distribution of predominant MDR patterns and AR genes in humans, pigs and environment……………………………………………………………………………...120

Table 4.1 MDR *Salmonella* serotypes from humans, pigs and their environment characterized for plasmids……………………………………………………………….155

LIST OF FIGURES

Figure 2.1 *Salmonella* prevalence among pigs at farm and slaughter………………………82

Figure 2.2 *Salmonella* prevalence in the environment at farm and slaughter……………….83

Figure 2.3 *Salmonella* prevalence among environmental samples at farm and slaughter.….84

Figure 2.4 Dendrogram showing genotypic similarity among *Salmonella* isolated from ABF systems at various stages of production……………………………………………………...85

Figure 2.5 Dendrogram showing genotypic similarity among *Salmonella* isolated from conventional system at various stages of productions………………………………………86

Figure 2.6 Dendrogram showing genotypic similarity among *Salmonella* isolated from conventional and ABF systems at various stages of production (Supplement)……………...87

Figure 3.1 Antimicrobial resistance profile of *Salmonella* isolates from humans, pigs and environment...122

Figure 3.2 Dendrogram showing genotypic similarity among *Salmonella* Derby isolated from humans, pigs and environment……………………………………………………..123

Figure 3.3 Dendrogram showing genotypic similarity among *Salmonella* Typhimurium isolated from humans, pigs and environment……..124

Figure 3.4 Dendrogram showing genotypic similarity among *Salmonella* serotypes isolated from humans, pigs and environment……………………………………………………..125

Figure 4.1 Plasmid restriction analysis with *EcoR*I enzyme of *Salmonella* serotypes…….157

Figure 4.2 Plasmid restriction of β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and their environment…………………………………………………......158

Figure 4.3 Multiplex PCR for plasmid replicon typing……………………………………160

Figure 4.4 Southern hybridization of bla_{CMY-2} gene in β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and their environment………………………………………161

1. CHAPTER I: Literature Review

1.1 Introduction

Salmonella is one of the most important zoonotic foodborne pathogen causing salmonellosis both in humans and animals worldwide (1). More than 2500 *Salmonella* serovars have been identified in the *Salmonella enterica* genus and all are considered capable of causing salmonellosis in humans (2, 3, 4). In humans, non-typhoidal salmonellosis is the most commonly reported bacterial foodborne illness worldwide and is responsible for large number of foodborne outbreaks in both developed and developing countries (5, 6). *Salmonella* infections also result in significant economic losses due to reduced productivity, lost wages and increased health care costs.

According to the Centers for Disease Control and Prevention (CDC), non-typhoidal salmonellosis in humans is the most commonly reported bacterial foodborne illness in the United States (7). In the US, there are an estimated 1.4 million cases, 55,961 hospitalizations and 1,351 deaths resulting in billions of dollars in losses each year (8). Among foodborne illness, *Salmonella* alone is responsible for a reported 1,000,000 cases due to food consumption only (9). It is important to note that the actual incidence of salmonellosis is estimated to be 38 times the number of reported cases (10). The CDC and Foodborne Diseases Active Surveillance Network (Food Net) highlights that salmonellosis is responsible not only for a large number of illnesses, but also for significant economic losses, estimated to range from $600 million to $3.5 billion each year in the United States (7, 8, 10).

Salmonella primarily inhabit the human and animal gastrointestinal tract causing mild to moderate self-limiting gastroenteritis disease called salmonellosis which does not require

antimicrobial treatment (11). Acute symptoms usually last for two to seven days and include diarrhea, nausea, abdominal pain, vomiting and fever. Severe and fatal illness is rare and mainly associated with impaired immune systems. However, in a minority of cases, non-typhoid salmonellosis may evolve to chronic disease resulting in localized infections, reactive arthritis, and even neurological and neuromuscular illnesses (12, 13).

1.2 *Salmonella* History, Morphology and Nomenclature

Salmonella was named after Dr. Daniel E. Salmon, a notable American veterinary pathologist. However, Theobald Smith was the actual discoverer of the type of bacterium (*Salmonella enterica* var. *choleraesuis*) in 1885. *Salmonella* are Gram-negative, rod-shaped, non-spore forming and facultative anaerobes. The size of the *Salmonella* varies with diameters ranging from 0.7 to 1.5 µm and lengths from 2 to 5 µm (14-16). They are chemoorganotropic, with an ability to metabolize nutrients by respiratory and fermentative pathways. *Salmonella* grows well in a wide range of environmental conditions, with a temperature range from 8°C to 45°C, pH range from 4 to 8 and water activities above 0.94. However, the optimal condition for growth is 37°C and a pH of 6.5 to 7.5. *Salmonella* are heat labile and can be inactivated at normal cooking temperature of above 70°C. In addition, *Salmonella* can tolerate up to 20% salt concentration. *Salmonella* can survive under extreme environmental conditions including freezing temperatures (from -23°C to -18°C) up to seven years (6, 17, 18). All members of this genus are motile by peritrichous flagella, except *S.* Pullorum and *S.* Gallinarum. In addition to peritrichous flagella, they are covered with surface pilli which are short hair like structures that are involved in cellular attachment (14, 16). The outer membrane of the cell wall of *Salmonella* is composed of various structurally

and functionally important molecules. One of these molecules is lipopolysaccharide (LPS) which is an important virulence factor for Gram-negative bacteria (19, 20).

Under laboratory conditions, *Salmonella* can be recovered from various sources in three steps including pre-enrichment in buffered peptone water (BPW) or lactose broth followed by enrichment in selective broth, such as Rasspaport- Vasilliadis (RV) broth, Selenite Cysteine Broth (SC), or tetrationate broth (TT). The final step is growth on a variety of selective media including Xylose Lysine Desoxycholate (XLD) agar, Brilliant Green agar (BGA), Xylose Lysine Tergitol-4 (XLT-4) and other chromogenic media. Identification and confirmation of *Salmonella* includes failure to hydrolyze urea on urea slants and production of acid and hydrogen sulfide gas from glucose fermentation in triple sugar iron agar (TSI) (6, 16).

The genus *Salmonella* is a member of the *Enterobacteriaceae* family and classified into two species: *Salmonella enterica* and *Salmonella bongori*. Formerly, *Salmonella bongori* was designated *S. enterica* subspecies V and thus commonly referred to as "subspecies V" (14, 15). *S. enterica* is further subdivided into six subspecies which are referred to by a Roman numeral and subspecies name (I, *enterica*; II, *salamae*; IIIa, *arizonae*; IIIb, *diarizonae*; IV, *houtenae* and VI, *indica*). The White-Kauffman scheme is currently used worldwide for serological identification of *Salmonella* serovars (21). Serotyping of *Salmonella* is performed based on surface antigens including somatic antigens (O), falgellar antigens (H) and capsular antigens (Vi). Apart from conventional serotyping, other methods based on fingerprint profile identification, Polymerized Chain Reaction (PCR), Microarray are also used to identify the *Salmonella* serotypes (22-24). More than 2500 *Salmonella*

serovars have been identified with the majority (99%) belonging to subspecies *S. enterica*, as shown in the Table 1.1(25). In addition, the majority of the serovars causing human salmonellosis belongs to this subspecies (2, 14, 15).

1.3 *Salmonella* in Humans, Pigs and Their Environment

Salmonella has a wide host range including both wild and domestic animals. This wide host range improves transmission of *Salmonella* infection to humans. Among all food sources, food of animal origin, especially contaminated poultry, beef and pork, serve as the major sources for human *Salmonella* outbreaks in the United States (26). In addition, contact with non-food sources such as contaminated water, direct contact with farm animals, pets and the environment also may result in human salmonellosis (12, 27). The non-typhoidal *Salmonella* (NTS) serotypes including *S*. Typhimurium, *S*. Heidelberg, *S*. Infantis, *S*. Muenchen, *S*. Anatum and *S*. Derby are commonly isolated from food animals, retail food products, and other environmental sources, and are responsible for a majority of foodborne human salmonellosis in the US (9, 28). In past decade, many *Salmonella* outbreaks in humans associated with pork have been reported worldwide, including in the United States (26, 29, 30). However, no outbreaks of human salmonellosis associated with pork have been reported in the Southeastern US where this work has taken place.

Humans and pigs have specific host adopted *Salmonella* serotypes including, *S*. Typhi, Paratyphi A and *S*. Cholerasuis respectively (26, 28). *S*. Cholerasuis was considered as the only disease causing strain in pigs. However, pigs are also susceptible to other non-host adopted strains especially *S*. Tyhpimurium which causes disease in nursery pigs (31). Occurrence and dissemination of *S*. Typhimurium serotype in pigs has significance from a

public health point of view as it is responsible for foodborne outbreaks in humans (32). The top ten most frequently reported *Salmonella* serovars to the CDC from humans and pigs are listed in Tables 1.2 and 1.3, with *S*. Tyhpimurium, *S*. Anatum, *S*. Infantis and *S*. Heidelberg being the most predominant (9, 28, 33).

The prevalence of *Salmonella* in farm animals in the US ranges from 1-6% and on swine farms from 1.4-33% (34-37). Studies have reported a higher prevalence in North Carolina, ranging from 16 to 29%. The prevalence of *Salmonella* in pigs and their environment also varies with farm type and production stage (38-40). The prevalence of *Salmonella* has been found to be higher at post-harvest stages at slaughter houses, ranging from 0% to 77%. Factors attributing to this higher rate of prevalence are believed to be due to transportation stress, cross contamination and the hygienic conditions at slaughter houses (40, 41). Higher prevalence of *Salmonella* at post-harvest stages has also been reported in the European Union (30, 42, 43).

The environment also serves as an important *Salmonella* reservoir due to dissemination of the pathogen into the environment by human and animal activities. Fecal shedding of *Salmonella* from swine result in contamination of surrounding environment which may act as a potential source as reservoirs (43, 44, 45). *Salmonella* can survive in the natural environment for weeks to several years depending on environmental conditions. Even though *Salmonella* may not multiply significantly in the natural environment, the environment still serves as potential source for *Salmonella* dissemination (6, 44, 46). Feed, water, the pen floor and lagoon frequently serve as important reservoirs for *Salmonella* at pre-harvest stages, while the lairage (resting area), slaughter robotics (carcass cutter/splitter)

and slaughter trucks are important reservoirs at post-harvest stages. It is evident that the environment plays a crucial role as reservoir in the transmission of *Salmonella* to pigs all along the production chain, either directly or indirectly (41). It is hypothesized that elimination of *Salmonella* from the environment is very difficult; therefore control measures should be made to reduce its occurrence and dissemination into food chain.

1.4 Antimicrobial Use in Food Animals and Humans

The commercial food animal industry uses antimicrobials for different reasons including treatment, control, prevention of disease, maintenance of herd health, and for growth promotion in the United States and the European Union for more than five decades (50, 51). The use of antimicrobials as growth promoters started after the discovery of the effect of sub-therapeutic doses of procaine penicillin and tetracycline on the enhancement of feed-to-weight ratio in swine, poultry and beef cattle (52). Thereafter, antimicrobials have been administered at sub-therapeutic doses continuously in swine, cattle and poultry feed to improve growth (53). Chlortetracycline was the first antimicrobial used as a feed additive. The Union of Concerned Scientists (UCS) estimated that over 11,200 metric tons of antimicrobials are used annually for nontherapeutic purposes alone in swine, cattle and poultry industry (54). The amount of antimicrobials used for nontherapeutic purposes is increasing every year and annual sales for antimicrobials used in humans and food animals are shown in Table 1.4 (55, 56).

In the US, the role of therapeutic and prophylactic antimicrobial use in food animals, development and propagation of AR bacterial populations, and their subsequent transmission to humans through the food chain is under extensive debate (57, 58). The European Union

banned the use of antimicrobials for growth promotion purposes in the food animals to reduce the threat of antimicrobial resistant pathogens to humans and animals (50). Similarly, growing concern over antimicrobial resistance from the public has been tremendous pressure on the US food animal industry to withdraw antimicrobials used for growth promotion. In addition, the World Health Organization (WHO) developed risk management strategies to reduce the development of antimicrobial resistance due to non-human antimicrobial use including antimicrobials used as growth promoters in food animals (59). The most common antimicrobials used as growth promoters that are also used in human medicine for treatment purposes including tetracyclines, cephalosporins and fluoroquinolones (51, 60). The WHO categorized antimicrobials used in humans based on their importance to treat diseases caused by different foodborne pathogens including *Salmonella*, *Campylobacter* and *Escherichia coli*. The most critically important antimicrobials used in human medicine are cephalosporins, including 3rd and 4th generations, and ciprofloxacin (61). The detailed list of critically important antimicrobials used in the humans is shown in Table 1.5 (59). The surveillance and monitoring of resistance by enteric pathogens including *Salmonella*, *Campylobacter*, *Escherichia coli*, *Shigella* and *Vibrio* to critically important antimicrobials used in humans and animals is monitored by National Antimicrobial Resistance Monitoring System (NARMS) in collaboration with the CDC, Food and Drug Administration (FDA) and United States Department of Agriculture (USDA).

1.5 Antimicrobial Use in Swine Industry and its Impact

The commercial swine industry in the US uses subtherapeutic doses of antimicrobials in feed to improve growth rate and to maintain pig performance in the presence of sub-

clinical diseases since the 1950's. The use of antimicrobials in feed is approved and regulated by FDA (53, 62). The amount (percent) of antimicrobials used in feed as additives varies at different stages of pig production including, starter pig feeds (85%), grower pig feeds (75%), finisher pig feeds (55%) and sow feeds (20%) (53). The antimicrobials approved by FDA as feed additives and their withdrawal periods are listed in the Table 1.6 including, apramycin, bacitracin methylene disalicylate (BMD), chlortetracycline, lincomycin, neomycin, oxytetracyline, penicillin, tiamulin, tylosin, virginiamycin (53). In addition to antimicrobials, chemotherapeutics (synthetic chemicals having antimicrobial activity) and minerals are also approved as feed additives includes; sulfamethazine, sulfathiazole, carbadox, roxarsone, arsanilic acid, zinc and copper (53, 62, 63).

The use of antimicrobials in swine has resulted in significant improvements in pig production in terms of reducing pig mortality, morbidity and subclinical diseases. In addition, studies have demonstrated that the use of antimicrobials has greater benefits in younger pigs, particularly at the nursery stage (53, 64, 65). Studies reported that the use of chlortetracyline in breeding and lactating sow's diet resulted in an improvement in sow's reproductive performance in terms of farrowing rate and litter size (66). The exact process by which antimicrobials enhance growth performance is yet to be explored. The possible modes of action of these antimicrobials as growth promoters include improved metabolic, nutritional and disease control effects (53).

The advantages of using antimicrobials in the swine industry and their impact on production rate have been demonstrated. However, the use of antimicrobials and their association to the development AR against clinically important antimicrobials used in human

medicine is under extensive debate (50). The ban of antimicrobials for growth purpose in pigs in the European Union has resulted in new challenges including increased use of antimicrobials used for treatment purposes (67). Emergence and persistence of AR foodborne pathogens due to routine use of antimicrobials in commercial swine production are a major public health concern (61). With growing consumer concern over AR, the demand for antimicrobial free (ABF) and organic products has increased over the past decade (68). No antimicrobials are administered either for treatment or growth purposes in both ABF and organic production. It is estimated that, there was a 58% increase of certified organic hogs and pigs between 2000-2005 (68). However, this alternative production system needs a higher health status and hygiene to combat against other clinical diseases caused by infectious viral and bacterial pathogens.

1.6 Antimicrobial Resistant *Salmonella* and its Public Health Concerns

Emergence and dissemination of drug resistant *Salmonella* strains in humans and food animals is a growing public health concern. Studies have demonstrated the occurrence and shedding of AR *Salmonella* strains from commercial pigs which use antimicrobials for therapeutic and non-therapeutic purposes (38, 61). However, studies have reported AR *Salmonella* from ABF and organic production systems in the absence of selection pressure (39, 40, 69). It is evident that the farm environment of commercial, ABF and organic production harbors AR *Salmonella* and plays a crucial role as a reservoir in transmission to pigs and humans (38, 69, 70). *Salmonella* isolated from human clinical cases, pigs and their environment exhibit wide spectrum antimicrobial resistance to commonly and critically important antimicrobials used in human and veterinary medicine (70, 71). According to the

2009 NARMS Report and other studies, the highest frequency of AR exhibited by *Salmonella* strains includes tetracycline, aminoglycosides, sulfisoxazole and β-lactams including 3rd and 4th generation cephalosporins (72-74). These 3rd and 4th generation cephalosporins are extensively used to treat severe clinical *Salmonella* infections, especially in children. Resistance to this group of antimicrobials by *Salmonella* strains of human and pig origin have been a major public health concern worldwide (75, 76).

Multidrug resistance (resistance to ≥ 3 antimicrobials; MDR) associated with different *Salmonella* serotypes from humans, pigs at various stages of production at farm, slaughter and their environment has been reported worldwide, including the US (43, 71, 77). However, their continued maintenance in the absence of selection pressure is especially concerning (40, 74). In the US, the percentage of reported non-typhoidal *Salmonella* isolates in humans and pigs that exhibit MDR was around 12 % and 30% from 1999-2010, respectively (72). According to the 2010 NARMS Report and other studies, the two most common MDR patterns observed among *Salmonella* Typhimurium are FIS STR TET and AMP CHL FIS STR TET. The penta-resistnat pattern (AMP CHL FIS STR TET) is specific to *S*. Typhimurium phage type DT104 and commonly associated with clinical salmonellosis and human foodborne outbreaks worldwide (75, 78, 79). This penta-resistant phenotype is often a component of higher-order MDR patterns that include resistance elements against the critically important fluoroquinolones and 3rd and 4th generation cephalosporins (77). Occurrence and dissemination of AR and MDR *Salmonella* in humans, pigs and their environment demands further molecular epidemiological studies to determine the exchange of AR pathogens between them.

1.7 Antimicrobial Resistance Mechanisms

Resistance to critically important antimicrobials by foodborne pathogens is an increasing health and economic problem (71). Bacterial pathogens evade the bactericidal and bacteriostatic effects of antimicrobials by altering their mechanism of action resulting in development of AR. There are four main mechanisms of resistance including destruction or inactivation of the binding site, mutation, production of enzymes, and efflux pump (80). β-lactams, which are considered to be critically important drugs in humans medicine, exhibit multiple mechanisms of resistance (81) including inactivation of antimicrobial agents, activation of antimicrobial efflux pumps, reduction of cell permeability to antimicrobials and alteration of the binding site of antimicrobials. This results in decreased therapeutic effectiveness of mainly penicillins and cephalosporins against bacterial pathogens. This type of resistance mechanism in *Salmonella* is mediated by the enzymes AmpC, TEM, Extended Spectrum Beta Lactamases enzymes (ESBLs) encoded by bla_{CMY-2}, bla_{TEM}, bla_{PSE} and CTX-M genes (82, 83). Another class of antimicrobials that has multiple mechanisms of resistance is aminoglycosides, which includes gentamicin, kanamycin and streptomycin. Aminoglycosides exhibit resistance by reduced uptake or decreased cell permeability, alterations at the ribosomal binding sites, or production of aminoglycoside modifying enzymes (84). The enzymes that mediate resistance are called aminoglycoside modifying enzymes and are encoded by the *aac*C, *aph*A and *aad*A/B gene which confer resistance to gentamicin, kanamycin and streptomycin respectively (84, 85).

Chlortetracylines are extensively used in the swine industry for growth and treatment purposes (58). The major antimicrobial resistance mechanism by which *Salmonella* confer

resistance to tetracylines and chloramphenicol is through active efflux mechanism. This is an energy dependent mechanism by the cell that actively pumps the drug molecules out of the cell cytoplasm (80, 86, 87). The genes commonly associated with tetracycline and chloramphenicol efflux pumps are *tet* families and *cml/ flo* genes respectively (88, 89). Resistance to quinolones is predominantly due to mutations. The mutations mainly occur in the genes encoding for the DNA gyrase enzymes which are required for *Salmonella* DNA replication. These mutations alter the binding site of antimicrobials and confer resistance to quinolones (80). However, plasmid mediated quinolones resistance (*qnr* plasmids) have been identified from different serovars of *Salmonella* from human clinical cases (90).

Horizontal gene transfer plays an important role in the rapid spread of AR determinants to susceptible bacterial population through conjugation, transduction and transformation (80, 90). Transformation is the process by which susceptible bacterial cells takes up naked DNA from the environment, while transduction involves transfer of genetic material containing AR determinants through bacteriophage. Conjugation requires cell to cell contact thorough sex pilli to transfer genetic material through mobile genetic elements including plasmids, transposons and integrons. This process has been well documented in the transfer of AR genes to *Salmonella* of human and pig origin (75, 80, 91). Integrons are mobile genetic elements containing resistance gene cassettes which carried either on plasmids or integrated into chromosome. Integrons have been identified both in Gram positive and Gram negative bacteria including *Salmonella* (1, 70, 76). Four classes of integrons have been identified based on the integrase gene sequence, with the class I integrons being most frequently identified among different *Salmonella* serotypes. *Salmonella*

Typhimirum DT104, which is commonly associated with foodborne outbreaks worldwide, carries class I integrons with five different resistance gene cassettes. Class II integrons have been detected only in a few *Salmonella* serotypes (76, 80, 92). Another important mobile genetic element involved in the rapid dissemination of AR determinants is plasmids. Plasmids carry resistance genes called R-plasmids and plasmid mediated resistance has been identified for the majority of clinically important antimicrobials, including quinolones (90, 93). In addition, studies have identified inter serovar exchange of plasmids encoding resistance to multiple antimicrobials (91). Different types of plasmids have been identified with Inc A/C plasmid encoding resistance to multiple antimicrobials including ESBL's being predominant among *S*. Typhimurium and *S*. Newport isolates (94).

Overall, there is a paucity of information regarding dissemination of AR *Salmonella* in pigs, environment and humans. Therefore, to identify different environmental reservoirs that aid in transmission of AR *Salmonella* in pigs all along the production we designed this study with following objectives: 1) To determine the dynamics of *Salmonella* in pigs reared in distinct production system and risk factors associated for its persistence at farm and slaughter. 2) To characterize and compare the isolates from pigs, environment and humans at phenotypic and genotypic level. 3) To sequence unique plasmids and its inter serovar transmission among humans, pigs and environment

References

1. Lynne AM, Dorsey LL, David DE, Foley SL. 2009. Characterisation of antibiotic resistance in host-adapted *Salmonella* enterica. Int. J. Antimicrob. Agents. 34:169-172.
2. Grassl G, Finlay B. 2008. Pathogenesis of enteric *Salmonella* infections. Curr. Opin. in Gastroenterol. 24:22-26.
3. Coburn B, Grassl GA, Finlay BB. 2007. *Salmonella*, the host and disease: a brief review. Immunol. Cell Biol. 85:112-118.
4. CDC. 2011. National enteric disease surveillance: *Salmonella* surveillance overview. Available online at http://www.cdc.gov/nationalsurveillance/PDFs/NationalSalmSurveillOverview_508.pdf.
5. Team Ee. 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011 has been published. Euro. Surveill. 18:20449.
6. WHO. 2002. Risk assessments of *Salmonella* in eggs and broiler chickens. Food and Agriculture Organization of the United Nations and World Health Organization, Geneva,Switzerland.
7. CDC. 2011. Centers for Disease Control and Prevention: Surveillance for foodborne disease outbreaks, United States, 2008. Morb. Mortal. Wkly. Rep. 60;1197-1202.

8. Scallan E, Hoekstra R, Angulo F, Tauxe R, Widdowson M, Roy S, Jones J, Griffin P. 2011. Foodborne illness acquired in the United States - Major pathogens. Emerg. Infect. Dis. 17:7-15.

9. CDC. 2009. *Salmonella* annual summary tables. Centers for Disease Control and Prevention, Atlanta, GA.

10. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV, Group EIPFW. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin. Infect. Dis. 38 Suppl 3:S127-134.

11. Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD. 2001. Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents. Chemother. 45:3647-3650.

12. Hoelzer K, Switt A, Wiedmann M. 2011. Animal contact as a source of human non-typhoidal *salmonellosis*. Vet. Res. 42:34.

13. Hamilton R, Hulsebus H, Akbar S, Gray J. 2012. Increased resistance to multiple antimicrobials and altered resistance gene expression in CMY-2-positive *Salmonella enterica* following a simulated patient treatment with ceftriaxone. Appl. Environ. Microbiol. 78:8062-8066.

14. Tindall B, Grimont P, Garrity G, Euzeby J. 2005. Nomenclature and taxonomy of the genus *Salmonella*. Int. J. of Syst. Evol. Microbiol. 55:521-524.

15. Brenner F, Villar R, Angulo F, Tauxe R, Swaminathan B. 2000. *Salmonella* nomenclature - Guest commentary. J. Clin. Microbiol. 38:2465-2467.

16. Hendriksen RS. 2003. A global *Salmonella* surveillance and laboratory support projectof the World Health Organization: Laboratory protocols level 1 training course isolation of *Salmonella* 4th Ed. Available online at http://www.antimicrobialresistance.dk/data/images/salmonella1_pdf.pdf.

17. Lianou A, Koutsoumanis KP. 2012. Strain variability of the biofilm-forming ability of *Salmonella enterica* under various environmental conditions. Int. J. Food. Microbiol. 160:171-178.

18. Alvarez-Ordóñez A, Valdés L, Bernardo A, Prieto M, López M. 2013. Survival of acid adapted and non-acid adapted *Salmonella* Typhimurium in pasteurized orange juice and yogurt under different storage temperatures. Food Sci. Technol. Int. DOI: 10.1177/1082013212455343

19. Rosenberger CM, Scott MG, Gold MR, Hancock RE, Finlay BB. 2000. *Salmonella* Typhimurium infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression. J. Immunol. 164:5894-5904.

20. Yoon H, Ansong C, Adkins JN, Heffron F. 2011. Discovery of *Salmonella* virulence factors translocated via outer membrane vesicles to murine macrophages. Infect. Immun. 79:2182-2192.

21. Popoff M, Bockemuhl J, Brenner F, Gheesling L. 2001. Supplement 2000 (no. 44) to the Kauffmann-White scheme. Res. Microbiol. 152:907-909.

22. Kim S, Frye J, Hu J, Fedorka-Cray P, Gautom R, Boyle D. 2006. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp enterica. J. Clin. Microbiol. 44:3608-3615.

23. Zou W, Lin WJ, Hise KB, Chen HC, Keys C, Chen JJ. 2012. Prediction system for rapid identification of S*almonella* serotypes based on pulsed-field gel electrophoresis fingerprints. J. Clin. Microbiol. 50:1524-1532.

24. Peterson G, Gerdes B, Berges J, Nagaraja TG, Frye JG, Boyle DS, Narayanan S. 2010. Development of microarray and multiplex polymerase chain reaction assays for identification of serovars and virulence genes in *Salmonella enterica* of human or animal origin. J. Vet. Diagn. Invest. 22:559-569.

25. Patrick A, Weill F-X. 2007. Antigenic formulae of the *Salmonella* serovars WHO Collaborating Centre for Reference and Research on Salmonella, p. 1-166.

26. CDC. 2007. The food borne outbreak online database (Food). Available online at http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx.

27. Hendriksen S, Orsel K, Wagenaar J, Miko A, van Duijkeren E. 2004. Animal-to-human transmission of *Salmonella* Typhimurium DT104A variant. Emerg. Infect. Dis. 10:2225-2227.

28. Foley SL, Lynne AM, Nayak R. 2008. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. J. Anim. Sci. 86:E149-162.

29. Scavia G, Ciaravino G, Luzzi I, Lenglet A, Ricci A, Barco L, Pavan A, Zaffanella F, Dionisi A. 2013. A multistate epidemic outbreak of *Salmonella* Goldcoast infection in

humans, June 2009 to March 2010: the investigation in Italy. Euro. Surveill. 18:23-31.

30. Wojcik O, Kjelso C, Kuhn K, Muller L, Jensen T, Kjeldsen M, Ethelberg S. 2012. *Salmonella* Typhimurium outbreak associated with smoked pork tenderloin in Denmark, January to March 2011. Scand. J. Infect. Dis. 44:903-908.

31. Dahl J, Wingstrand A, Nielsen B, Baggesen D. 1997. Elimination of *Salmonella* Typhimurium infection by the strategic movement of pigs. Vet. Rec. 140:679-681.

32. Helms M, Ethelberg S, Molbak K, Grp DS. 2005. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emerg. Infect. Dis. 11:859-867.

33. CDC. 2011. Foodborne Diseases Active Surveillance Network (FoodNet): Tables and Figures—2011 Preliminary Data. Available online at http://www.cdc.gov/foodnet/data/trends/tables/table5.html.

34. Dargatz D, Fedorka-Cray P, Ladely S, Kopral C, Ferris K, Headrick M. 2003. Prevalence and antimicrobial susceptibility of *Salmonella* spp. isolates from US cattle in feedlots in 1999 and 2000. J. Appl. Microbiol. 95:753-761.

35. Davies PR, Bovee FG, Funk JA, Morrow WE, Jones FT, Deen J. 1998. Isolation of *Salmonella* serotypes from feces of pigs raised in a multiple-site production system. J. Am. Vet. Med. Assoc. 212:1925-1929.

36. Rodriguez A, Pangloli P, Richards H, Mount J, Draughon F. 2006. Prevalence of *Salmonella* in diverse environmental farm samples. J. Food Prot. 69:2576-2580.

37. Barber DA, Bahnson PB, Isaacson R, Jones CJ, Weigel RM. 2002. Distribution of *Salmonella* in swine production ecosystems. J. Food. Prot. 65:1861-1868.

38. Dorr P, Tadesse D, Zewde B, Fry P, Thakur S, Gebreyes W. 2009. Longitudinal study of *Salmonella* dispersion and the role of environmental contamination in commercial swine production systems. Appl. Environ. Microbiol. 75:1478-1486.

39. Thakur S, Tadesse D, Morrow M, Gebreyes W. 2007. Occurrence of multidrug resistant *Salmonella* in antimicrobial-free (ABF) swine production systems. Vet. Microbiol. 125:362-367.

40. Gebreyes W, Thakur S, Morrow W. 2006. Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant *Salmonella* in antimicrobial-free and conventional pig production. J. Food Prot. 69:743-748.

41. Hurd H, Gailey J, McKean J, Rostagno M. 2001. Rapid infection in market-weight swine following exposure to a *Salmonella* Typhimurium-contaminated environment. Am. J. Vet. Res. 62:1194-1197.

42. Baggesen DL, Sandvang D, Aarestrup FM. 2000. Characterization of *Salmonella enterica* serovar Typhimurium DT104 isolated from Denmark and comparison with isolates from Europe and the United States. J. Clin. Microbiol. 38:1581-1586.

43. Sandvang D, Jensen LB, Baggesen DL, Baloda SB. 2000. Persistence of a *Salmonella enterica* serotype Typhimurium clone in Danish pig production units and farmhouse environment studied by pulsed field gel electrophoresis (PFGE). FEMS Microbiol. Lett. 187:21-25.

44. Baloda SB, Christensen L, Trajcevska S. 2001. Persistence of a *Salmonella enterica* serovar Typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. Appl. Environ. Microbiol. 67:2859-2862.

45. Rajic A, Keenliside J, McFall M, Deckert A, Muckle A, O'Connor B, Manninen K, Dewey C, McEwen S. 2005. Longitudinal study of *Salmonella* species in 90 Alberta swine finishing, farms. Vet. Microbiol. 105:47-56.

46. Baloda SB, Christensen L, Trajcevska S. 2001. Persistence of a *Salmonella enterica* serovar Typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. Appl. Environ. Microbiol. 67:2859-2862.

47. Swanenburg M, van der Wolf P, Urlings H, Snijders J, van Knapen F. 2001. *Salmonella* in slaughter pigs: the effect of logistic slaughter procedures of pigs on the prevalence of *Salmonella* in pork. Int. J. Food Microbiol. 70:231-242.

48. Funk J, Gebreyes W. 2004. Risk factors associated with *Salmonella* prevalence on swine farms. J. Swine Health Prod. 12:246-251.

49. van Hoek AH, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K, Aarts HJ, Heres L. 2012. A quantitative approach towards a better understanding of the dynamics of *Salmonella* spp. in a pork slaughter-line. Int. J. Food. Microbiol. 153:45-52.

50. Marshall B, Levy S. 2011. Food animals and antimicrobials: Impacts on human health. Clin. Microbiol. Rev. 24:718-+.

51. Gorbach S. 2001. Antimicrobial use in animal feed - Time to stop. N. Engl. J. Med. 345:1202-1203.

52. Stokstade E, Jukes T. 1950. Further observations on the animal protein factor. Proc. Soc. Exp. Biol. and Med. 73:523-528.

53. Cromwell GL. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. 13:7-27.

54. Mellon M, Benbrook C, Benbrook KL. 2001. Hogging it: estimates of antimicrobial abuse in livestock. UCS Publications., Cambridge, MA.

55. FDA. 2012. Drug use review. Available online at http://www.fda.gov/downloads/Drugs/DrugSafety/InformationbyDrugClass/UCM319435.pdf

56. FDA. 2011. Summary report on antimicrobials sold or distributed for use in food-producing animals. Available online at http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf.

57. Alali WQ, Scott HM, Christian KL, Fajt VR, Harvey RB, Lawhorn DB. 2009. Relationship between level of antibiotic use and resistance among *Escherichia coli* isolates from integrated multi-site cohorts of humans and swine. Prev. Vet. Med. 90:160-167.

58. Quintana-Hayashi MP, Thakur S. 2012. Longitudinal study of the persistence of antimicrobial-resistant *Campylobacter* strains in distinct swine production systems on farms, at slaughter, and in the environment. Appl. Environ. Microbiol. 78:2698-2705.

59. WHO. 2007. Critically important antimicrobials for human medicine: categorization for the development of risk management strategies to contain antimicrobial resistance due to non-human antimicrobial use.

60. Baker R. 2006. Health management with reduced antibiotic use - the U.S. experience. Anim. Biotechnol. 17(2):195-205.

61. Aarestrup FM, Oliver Duran C, Burch DG. 2008. Antimicrobial resistance in swine production. Anim. Health. Res. Rev. 9:135-148.

62. Jacela J, DeRouchey J, Tokach M, Goodband R, Nelssen J, Renter D, Dritz S. 2009. Feed additives for swine: Fact sheets - acidifiers and antibiotics. J. Swine Health Prod. 17:270-275.

63. Jacela J, DeRouchey J, Tokach M, Goodband R, Nelssen J, Renter D, Dritz S. 2010. Feed additives for swine: Fact sheets - high dietary levels of copper and zinc for young pigs, and phytase. J. Swine Health Prod. 18:87-89.

64. Zimmerman D. 1986. Role of subtherapeutic levels of antimicrobials in pig production. J. Anim. Sci. 62(3):6-17.

65. Gaskins HR, Collier CT, Anderson DB. 2002. Antibiotics as growth promotants: mode of action. Anim. Biotechnol. 13:29-42.

66. Alexopoulos C, Fthenakis GC, Burriel A, Bourtzi-Hatzopoulou E, Kritas SK, Sbiraki A, Kyriakis SC. 2003. The effects of the periodical use of in-feed chlortetracycline on the reproductive performance of gilts and sows of a commercial pig farm with a history of clinical and subclinical viral and bacterial infections. Reprod. Domest. Anim. 38:187-192.

67. Millet S, Maertens L. 2011. The European ban on antibiotic growth promoters in animal feed: from challenges to opportunities. Vet. J. 187:143-144.

68. USDA. 2009. Organic agriculture: organic market overview. US Departmentof Agriculture, Economic Research Service, Washington, DC. http://www.ers.usda.gov/topics/natural-resources-environment/organic-agriculture/organic-market-overview.aspx#.Ucw9oCIkA7V.

69. Jensen AN, Dalsgaard A, Stockmarr A, Nielsen EM, Baggesen DL. 2006. Survival and transmission of *Salmonella enterica* serovar Typhimurium in an outdoor organic pig farming environment. Appl. Environ. Microbiol. 72:1833-1842.

70. Van TT, Nguyen HN, Smooker PM, Coloe PJ. 2012. The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in South East Asia. Int. J. Food. Microbiol. 154:98-106.

71. Glenn L, Lindsey R, Folster J, Pecic G, Boerlin P, Gilmour M, Harbottle H, Zhao S, McDermott P, Fedorka-Cray P, Frye J. 2013. Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Canada. Microb. Drug Resist. 19:175-184.

72. NARMS. 2010. National Antimicrobial Resistance Monitoring System:executive report 2010, Atlanta, Georgia, U.S. Department of health and human services, CDC.

73. Gebreyes W, Altier C. 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp *enterica* serovar Typhimurium isolates from swine. J. Clin. Microbiol. 40:2813-2822.

74. Keelara S, Scott HM, Morrow WM, Gebreyes WA, Correa M, Nayak R, Stefanova R, Thakur S. 2013. Longitudinal study comparing the distribution of phenotypically and

genotypically similar antimicrobial resistant *Salmonella* serovars between pigs and their environment in two distinct swine production systems. Appl. Environ. Microbiol. doi: 10.1128/AEM.01419-13

75. Gebreyes W, Thakur S, Dorr P, Tadesse D, Post K, Wolf L. 2009. Occurrence of spvA virulence gene and clinical significance for multidrug-resistant *Salmonella* strains. J. Clin. Microbiol. 47:777-780.

76. Vo A, van Duijkeren E, Gaastra W, Fluit A. 2010. Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolates from Vietnam. Plos One 5:e9440

77. Patchanee P, Zewde B, Taclesse D, Hoet A, Gebreyes W. 2008. Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg isolated from humans and animals. Foodborne Pathog. Dis. 5:839-851.

78. Helms M, Ethelberg S, Mølbak K, Group DS. 2005. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emerg. Infect. Dis. 11:859-867.

79. Medalla F, Hoekstra R, Whichard J, Barzilay E, Chiller T, Joyce K, Rickert R, Krueger A, Stuart A, Griffin P. 2013. Increase in resistance to ceftriaxone and nonsusceptibility to ciprofloxacin and decrease in multidrug resistance among *Salmonella* strains, United States, 1996-2009. Foodborne Pathog. Dis. 10:302-309.

80. Harbottle H, Thakur S, Zhao S, White D. 2006. Genetics of antimicrobial resistance. Anim. Biotechnol. 17:111-124.

81. Bradford PA. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14:933-951.

82. Sefton A. 2002. Mechanisms of antimicrobial resistance - Their clinical relevance in the new millennium. Drugs 62:557-566.

83. McDermott P, Walker R, White D. 2003. Antimicrobials: Modes of action and mechanisms of resistance. Int. J. Toxicol. 22:135-143.

84. Kotra L, Haddad J, Mobashery S. 2000. Aminoglycosides: Perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob. Agents Chemother. 44:3249-3256.

85. Randall L, Cooles S, Osborn M, Piddock L, Woodward M. 2004. Antibiotic resistance genes, integrons and multiple antibiotic resistance in thirty-five serotypes of *Salmonella enterica* isolated from humans and animals in the UK. J. Antimicrob. Chemother. 53:208-216.

86. Okusu H, Ma D, Nikaido H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. 178:306-308.

87. Williams J. 1996. Drug efflux as a mechanism of resistance. Br. J. Biomed. Sci. 53:290-293.

88. Butaye P, Cloeckaert A, Schwarz S. 2003. Mobile genes coding for efflux-mediated antimicrobial resistance in Gram-positive and Gram-negative bacteria. Int. J. Antimicrob. Agents. 22:205-210.

89. Chopra I, Roberts M. 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Revi. 65:232-260.

90. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. 2009. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin. Microbiol. Rev. 22:664-689.

91. Gebreyes W, Thakur S. 2005. Multidrug-resistant *Salmonella enterica* serovar Muenchen from pigs and humans and potential interserovar transfer of antimicrobial resistance. Antimicrob. Agents Chemother. 49:503-511.

92. Rodriguez I, Martin M, Mendoza M, Rodicio M. 2006. Class 1 and class 2 integrons in non-prevalent serovars of *Salmonella enterica*: structure and association with transposons and plasmids. J. Antimicrob. Chemother. 58:1124-1132.

93. Gay K, Robicsek A, Strahilevitz J, Park C, Jacoby G, Barrett T, Medalla F, Chiller T, Hooper D. 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. Clin. Infect. Dis. 43:297-304.

94. Lindsey R, Fedorka-Cray P, Frye J, Meinersmann R. 2009. Inc A/C Plasmids are prevalent in multidrug-resistant *Salmonella enterica* isolates. Appl. Environ. Microbiol. 75:1908-1915.

Table 1.1 Number of *Salmonella* serovars per species and subspecies.

***Salmonella* species and subspecies**	**Number of Serovars**
S. enterica subsp. e*nterica* (I)	1,531
S. enterica subsp. s*alamae* (II)	505
S. enterica subsp. *arizonae* (IIIa)	99
S. enterica subsp. d*iarizonae* (IIIb)	336
S. enterica subsp. *houtenae* (IV)	73
S. enterica subsp. *indica* (VI)	13
S. bongeri (V)	22
Total	2,579

Reference: **Patrick A, Weill FX**. 2007. Antigenic Formulae of the *Salmonella* serovars WHO Collaborating Centre for Reference and Research on *Salmonella*, p. 1-166.

Table 1.2 The top 10 most frequently reported *Salmonella* serovars from humans, 2011.

Rank	***Salmonella* serotypes**	**Number of cases**	**Incidence per 100,000 persons**
1	*S*. Enteritidis	1,413	3
2	*S*. Typhimurium	976	2.07
3	*S*. Newport	955	2.03
4	*S*. Javiana	739	1.57
5	*S*. I 4,[5],12:i:-	312	0.66
6	*S*. Muenchen	198	0.42
7	*S*. Heidelberg	167	0.35
8	*S*. Montevideo	149	0.32
9	*S*. Infantis	124	0.26
10	*S*. I 13,23:b:-	120	0.25

References: CDC. 2009. *Salmonella* Annual summary tables. Centers for Disease Control and Prevention, Atlanta, GA.

Table 1.3 The top 10 most frequently reported *Salmonella* serovars from pigs, 2011.

Rank	*Salmonella* serotype	Number of cases	Percent of Total (N=339)
1	*S*. Derby	48	14.2
2	*S*. Typhimurium var. 5-	36	10.6
3	*S*. Agona	32	9.4
4	*S*. Infantis	27	8.0
5	*S*. Anatum	24	7.1
6	*S*. Johannesburg	21	6.2
7	*S*. Typhimurium	18	5.3
8	*S*. Berta	11	3.2
9	*S*. Rough, mucoid, and/or nonmotile	9	2.7
10	*S*. Braenderup	8	2.4

References: CDC. 2011. Foodborne Diseases Active Surveillance Network (FoodNet): Tables and Figures—2011 Preliminary Data. Available online at http://www.cdc.gov/foodnet/data/trends/tables/table5.html

Table 1.4 Annual sales for antimicrobials used in humans and food animals, 2011.

Antimicrobial Class	Animal Sales (kgs)	Human Sales (kgs)
Aminoglycosides	214,895	6,485
Cephalosporins	26,611	496,910
Ionophores	4,123,259	0
Lincosamides	190,101	71,455
Macrolides	582,836	164,028
Penicillins	880,163	1,460,421
Sulfas	371,020	481,664[a]
Tetracyclines	5,642,573	113,832
Other	1,510,572[b]	494,381[c]
Total	12,031,458	3,289,176

[a] Includes sulfas and TMP

[b] Includes aminocoumarins, amphenicols, diaminopyrimidines, fluoroquinolones, glycolipids, pleuromutilins, polypeptides, quinoxalines, and streptogramins

[c] Includes quinolones, nitromidazoles, carbapenems/penems, oxalozolidines, monobactams, lipopeptides, ketolides, and streptogrammins.

Reference: FDA. 2011. Summary report on antimicrobials sold or distributed for use in food-producing animals. Available online at http://www.fda.gov/downloads/ForIndustry/UserFees/AnimalDrugUserFeeActADUFA/UCM338170.pdf

FDA. 2012. Drug use review. Available online at http://www.fda.gov/downloads/Drugs/DrugSafety/InformationbyDrugClass/UCM319435.p

Table 1.5 WHO categories of antimicrobials according to their importance in human medicine.

Class	Antimicrobials		
	Critically Important	**Highly Important**	**Important**
Amidinopenicillins	---	mecillinam	---
Aminoglycosides	amikacin, arbekacin, gentamicin, netilmicin, tobramycin, streptomycin	kanamycin, neomycin, spectinomycin	---
Amphenicols	---	chloramphenicol, thiamphenicol	---
Ansamycins	rifabutin, rifampin, rifaximin	---	---
Carbapenems/penems	ertapenem, faropenem, imipenem, meropenem	---	---
1st/2nd generation Cephalosporins	---	cefaclor, cefamandole, cephalexin, cephalothin, cephradine, loracarbef	---
3rd/4th generation Cephalosporins	cefixime, cefoperazone, cefoperazone/sulbactam, cefotaxime, cefpodoxime, ceftazidime, ceftizoxime, ceftriaxone, cefepime, cefoselis, cefpirome	---	---
Cephamycins	---	cefotetan, cefoxitin	---
Cyclic polypeptides	---	---	bacitracin
Glycopeptides	teicoplanin, vancomycin	---	---
Lincosamides	---	---	lincomycin
Macrolides/ketolides	azithromycin, clarithromycin, roxithromycin, spiramycin, telithromycin	---	---

Table 1.5 (continued)

Nitroimidazoles	---	---	metronidazole, tinidazole
Oxazolidinones	Linezoilid	---	
Penicillins	ampicillin, ampicillin/sulbactam, amoxicillin, amoxicillin/clavulanate, penicillin G, penicillin V, piperacillin, piperacillin/tazobactam, ticarcillin, ticarcilling/clavulanate	cloxacillin, dicloxacillin, flucloxacillin, oxacillin, nafcillin	---
Polymyxins	---	colistin, polymyxin B	---
Quinolones	cinoxacin, nalidixic acid, pipemidic acid, ciprofloxacin, enoxacin, gatifloxacin, gemifloxacin, levofloxacin, lomefloxacin, moxifloxacin, norfloxacin,	---	---
Sulfonamides, DHFR	---	para-aminobenzoic acid, pyrimethamine, sulfadiazine, sulfapyridine, sulfisoxazole, trimethroprim	---
Sufones	---	dapsone	---
Tetracyclines	---	chlorotetracycline, oxytetracycline, tetracycline	---

Table 1.5 (continued)

Reference: WHO. 2007. Antimicrobials for Human Medicine: Categorization for the Development of Risk Management Strategies to contain Antimicrobial Resistance due to Non-Human Antimicrobial Use. Report of the Second WHO Expert Meeting Copenhagen, 29–31 May 2007. Available online at http://www.who.int/foodborne_disease/resistance/antimicrobials_human.pdf.

Table 1.6 FDA approved antimicrobials for treatment and growth promotion in swine.

Antimicrobial	Usage	Dose (g/ton)	Withdrawl Period (days)
Bacitracin zinc	growth promotion	10-50	0
Bambermycin	growth promotion	2-4	0
BMD	growth promotion	10-30	0
	grow-finish: control of swine dysentery	250	
	sows: control of clostridial enteritis in suckling piglets	250	
BMD + CTC	growth promotion	10-30 + 400	0
	treatment of bacterial enteritis and pneumonia	10-30 + 400	
Carbadox	growth promotion	10-25	42
	control of swine dysentery and salmonellosis	50	
CTC	growth promotion	10-50	voluntary withdrawal
	reduction of jowl abscesses	50-100	
	Sows: control of leptospirosis	400	
	control of proliferative enteropathies	10 mg/lb/d	
CTC + sufathiazole + penicillin	reduction of abscesses, treatment of bacterial enteritis, maintenance of weight gain in the presence of rhinitis	100 + 100 + 50	7

Table 1.6(continued)

Florfenicol	control of bacterial respiratory disease	182	13
Lincomycin	growth promotion	20	0
	control of swine dysentery and ileitis	40-100	
	reduce severity of mycoplasmal pneumonia	200	
Neomycin + OTC	growth promotion	Oct-50	5
	treatment of bacterial enteritis and pneumonia	10 mg/lb/d	
	control and treatment of leptospirosis in breeders	10 mg/lb/d	
Neomycin	treatment and control of bacterial enteritis	10 mg/lb/d	3
OTC	growth promotion	Oct-50	0
	treatment of bacterial enteritis and pneumonia	10 mg/lb/d	
	Sows: control of leptospirosis	10 mg/lb/d	

Table 1.6 (continued)

OTC + carbadox	treatment of bacterial enteritis and pneumonia	10-25 + 10mg/lb/d	42
OTC + neomycin	prevention and treatment of bacterial enteritis and dysentery, maintenance of weight gain in the presence of atrophic rhinitis	50-150 + 35-140 mg/lb/d	10
Tiamulin	control of dysentery and ileitis	35	2
	treatment of dysentery	200	7
Tiamulin + CTC	control of dysentery, treatment of bacterial enteritis and pneumonia	35 + 400	2
Tilmicosin	control of respiratory disease	181-363	7
Tylosin	growth promotion	10-100	0
	control of dysentery and ileitis	40-100	
Virginiamycin	growth promotion	10-May	0
	control of dysentery	25	
	treatment of dysentery	100	

Table 1.6 (continued)

BMD: Bacitracin methylene disalicylate; CTC: Chlorotetracycline; OTC: Oxytetracycline

Adapted from Jacela JA, DeRouchey JM, Tokach MD, Goodband RD, Nelssen JL, Renter DG, Dritz SS. 2009. Feed additives for swine: fact sheets – acidifiers and antibiotics. J. Swine Health Prod. 17: 270-275

2. CHAPTER II: Longitudinal study of distribution of similar antimicrobial resistant *Salmonella* serovars in pigs and their environment in two distinct swine production systems

Presented here is the manuscript titled "Longitudinal study of distribution of similar antimicrobial resistant *Salmonella* serovars in pigs and their environment in two distinct swine production systems", published the present year (2013) in the journal of *Applied and Environmental Microbiology*, vol. 79(17), pgs. 2698-2705. Additional data are presented in the Appendix.

2.1 Abstract

The aim of this longitudinal study was to determine and compare the prevalence and genotypic profile of antimicrobial resistant (AR) *Salmonella* isolates from pigs reared in antimicrobial free (ABF) and conventional production systems at farm, slaughter and in their environment. We collected 2,889 pig fecal and 2,122 environmental samples (feed, water, soil, lagoon, truck and floor swabs) from 10 conventional and eight ABF longitudinal cohorts at different stages of production (farrowing, nursery, finishing) and slaughter (post-evisceration, post-chill and mesenteric lymph nodes; MLN). In addition, we collected 1,363 carcass swabs and 205 lairage and truck samples at slaughter. A total of 1,090 *Salmonella* isolates was recovered from the samples; these were isolated with a significantly higher prevalence in conventionally reared pigs (4.0%; n=66) and their environment (11.7%; n=156) compared to the ABF pigs (0.2%; n=2) and environment (0.6%; n=5) ($P < 0.001$). *Salmonella* was isolated from all the stages at slaughter, including the post chill step, in the

two production systems. *Salmonella* prevalence was significantly higher in MLN extracted from conventional carcasses than those extracted from ABF carcasses ($P < 0.001$). We identified a total of 24 different serotypes, with *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* serovar Anatum, *Salmonella enterica* serovar Infantis, and *Salmonella enterica* serovar Derby being predominant. The highest frequency of antimicrobial resistance (AR) was exhibited to tetracycline (71%), sulfisoxazole (42%) and streptomycin (17%). Multidrug resistance (resistance to ≥ 3 antimicrobials; MDR) was detected in 27% (n=254) of the *Salmonella* isolates from the conventional system. Our study reports a low prevalence of *Salmonella* in both production systems in pigs on farms, while a higher prevalence was detected among the carcasses at slaughter. The dynamics of *Salmonella* prevalence in pigs and carcasses were reciprocated in the farm and slaughter environment, clearly indicating an exchange of this pathogen between the pigs and their surroundings. Furthermore, the phenotypic and genotypic fingerprint profile results underscore the potential role played by environmental factors in dissemination of AR *Salmonella* to pigs.

2.2 Introduction

Salmonella is a major bacterial foodborne pathogen causing infection in both humans and animals (1). In the United States, *Salmonella* is responsible for the highest number of foodborne related illnesses with a reported 1.4 million illnesses, 15,000 hospitalizations and deaths of more than 500 people each year (2). It is important to note that the actual incidence of salmonellosis is estimated to be 38 times the number of reported cases (3). Emergence and persistence of antimicrobial resistant (AR) foodborne pathogens due to routine use of

antimicrobials for therapeutic, preventive and growth purposes in conventional swine production is a major public health concern (4). Multidrug resistant (MDR) *Salmonella* strains, exhibiting resistance to third generation cephalosporins, have been reported in commerical pigs (4-7). With growing consumer concerns over AR in bacterial pathogens from commercial swine that are given antimicrobials for prophylaxis and treatment, the demand for antimicrobial free (ABF) and organic products has increased over the past decade (8). However, previous studies have highlighted the occurrence of MDR *Salmonella* in both ABF and organic food animal production despite the apparent absence of antimicrobial selection pressure (7, 9, 10).

The prevalence of *Salmonella* in swine farms in the US ranges from 1.4 to 33% (11-13). North Carolina is the second largest pork producing State in the US next to Iowa, with a 14.4% contribution to the national inventory (19). Studies in swine production systems have been conducted in North Carolina to report *Salmonella* prevalence (6, 7, 9). Interestingly, a higher *Salmonella* prevalence (16 to 29%) has been reported in the swine farm environment than in fecal samples within the same farm (12). *Salmonella* prevalence at the processing plant ranges from 0% to 77% (14, 15). In these studies, higher prevalence of *Salmonella* at slaughter was believed to be due to transportation stress, cross contamination and the hygienic condition of the slaughter facility. Phenotypic and genotypic analyses have shown that the environment and pre-slaughter handling, such as transport and lairage, play a significant role in the dissemination of this pathogen in pigs (14, 16-18). It is quite evident that the environment plays a crucial role as a reservoir in transmission of AR pathogens to pigs all along the production chain, either directly or indirectly (6, 12, 16).

Genotyping of *Salmonella* using pulsed field gel electrophoresis (PFGE) has been found to be effective in epidemiological studies for identifying different environmental factors as important in contributing to the dynamics of pathogen transmission (6, 19, 20). However, there is a paucity of information regarding the role of the environment in dissemination of AR *Salmonella* at farm versus slaughter in ABF and conventional systems. To the authors' knowledge, no longitudinal study has been conducted along the entire production chain from farrowing to slaughter to compare the prevalence, antimicrobial resistance and genotypic diversity of *Salmonella* among swine reared in ABF and conventional production systems and their environment. The objectives of this study were to: i) determine *Salmonella* prevalence and serotype distribution in swine and their environment in two distinct swine production systems at farm and slaughter, ii) compare the AR profiles of isolates from swine and their environment, and iii) evaluate the genotypic diversity and/or similarity among *Salmonella* isolates from swine and their environment along the production chain.

2.3 Materials and methods

2.3.1 Study design and sample source

In this longitudinal study design, a total of eight cohorts of ABF and ten cohorts of conventionally raised pigs were sampled in eight ABF and 30 conventional farms in North Carolina. In the conventional systems, pig cohorts flowed through 3 different farms at different stages of production (i.e., 10 cohorts times 3 farms per cohort = 30 farms). The conventional farms belonged to two different large-scale companies while the ABF farms

were owned by individual swine producers. In the conventional system, pigs were reared indoors and followed an all-in-all-out (AIAO) production system. The purpose of an AIAO system is to reduce disease transmission from one growth stage to another (21) and in order to do so the pigs were grouped together based on age, weight and production stage and moved from one location to another at end of each stage of production (i.e., farrowing, nursery and finishing stages).

Trucks were used to transport pigs from one farm to the next in line. Trucks that ferried pigs were washed and cleaned before they arrived on a farm to load pigs. In the ABF production system, pigs were housed outdoors on agricultural land and had access to the ambient environment. Pigs under the ABF production system were given non pelleted feed while the conventionally reared pigs were provided the pelleted form.

All the stages of pig life cycle under the ABF production system were at the same location but involved rotation to different pastures. The conventionally raised pigs were given antimicrobials for growth, prophylaxis and therapeutic purposes, whereas ABF pigs raised to slaughter age were not given antimicrobials for any purpose; that is, in keeping with ethical standards any ABF pig requiring treatment with antimicrobials for bacterial infection was provided such care and subsequently removed from the herd.

Sample size was calculated based on type I ($\alpha = 0.05$) and type II ($\beta = 0.20$) allowable errors and it was estimated that 27 to 35 pigs needed to be sampled to detect a statistically significant difference in the proportion of *Salmonella* positive pigs in the two production systems. We purposely selected healthy pigs at the farrowing farm with the aim of sampling the same cohort of 35 pigs at slaughter.

2.3.2 Sampling on farm

During each sequential visit, samples were collected from the ABF and conventional pig cohorts and their environment. Sampling was carried out from October 2008 to December 2010 at various stages of production, including once at farrowing (7-10 day old), twice at each of nursery (4 and 7 weeks of age) and finishing stages (16 and 26 weeks of age), and finally once at slaughter. During the farrowing stage, a cohort of 35 healthy piglets per farm (4 piglets/sow) were selected and ear tagged for identification; subsequently, sampling followed the same cohort of pigs at different sampling stages during farm and slaughter stages. Fresh fecal samples (10 g) were collected from piglets using sterile fecal loops (Webster Veterinary, Devens, MA) and from their respective sows, using sterile gloves to aid in the determination of the transmission of *Salmonella* from sows to piglets at birth. Similarly, fecal samples were collected from the ear tagged pigs twice at each of nursery and finishing stages using gloved hands. Environmental samples also were collected at every stage of sampling to determine the role played by the environment as a reservoir and in the transmission of *Salmonella* to/from and among the pigs. Environmental sampling at ABF and conventional farms consisted of five samples each of water, feed, soil and barn floor swabs. All of the ABF farm environmental samples were collected outdoors whereas the conventional environmental samples were collected indoors, except for soil samples which were collected from outside the barns. In addition to these environmental samples, lagoon (repository of waste water draining from the barns) and inter-farm truck samples were collected only at conventional farms. Since trucks form an integral part of the pig environment, we sampled the four corners and the center of the truck floor by swabs pre-

soaked with buffered peptone water (BPW; Difco, Becton Dickinson, Sparks, MD, USA). Similarly, the barn floor swab samples from conventional farms and the inside of hoop structures in ABF farms were collected. Overall, we collected a total of 2,889 fecal (ABF, 1,239; conventional, 1,650), 450 feed (ABF, 200; conventional, 250), 450 floor swabs (ABF, 200; conventional, 250), 449 soil (ABF, 199; conventional, 250), 448 water (ABF, 198; conventional, 250), 245 lagoon (only conventional) and 80 inter-farm truck (only conventional) samples from eight ABF and 30 (representing 10 cohorts of pigs) conventional farms and their environment. Samples were transported to the laboratory on ice at 4°C and processed immediately upon arrival.

2.3.3 Sampling at slaughter facility

Conventional pigs were transported to a large scale slaughter plant (9,000 pigs/day) which had a blast chilling facility (-30°C) to quickly freeze the carcasses. The ABF pigs were transported to two smaller scale slaughter plants (250 pigs/day), each of which had an overnight chilling facility (4°C) to freeze the carcasses. These smaller scale plants slaughtered only ABF pigs. At slaughter, we collected carcass swabs from the same cohort of pigs at two stages; specifically, the post-evisceration and post-chilling stages. At the post-evisceration stage, we collected samples of mesenteric lymph node (MLN) from the pigs. Carcass swab samples were collected by wiping at three different positions (jowls, belly, and ham) on each carcass using the USDA-recommended method (22). Environmental samples from the floor of the truck transporting the pigs to the processing plant and lairage floor samples were collected and processed for *Salmonella* isolation. A total of 455 MLN (ABF, 184; conventional, 271), 454 post-evisceration carcasses swab (ABF, 182; conventional,

272), 454 post-chill carcasses swab (ABF, 199; conventional, 255), 130 lairage floor swab (ABF, 80; conventional, 50) and 75 truck floor swab (ABF, 35; conventional, 40) samples were collected. Samples were transported to the laboratory on ice and processed within three hours of collection.

2.3.4 *Salmonella* isolation and confirmation

Isolation and confirmation were performed as previously described (7, 9). Briefly, the samples were pre-enriched by adding 90ml of buffered peptone water (BPW) (Difco, Becton Dickinson) to cups containing either fecal or environment samples, whereas 30 ml of BPW was added to each bag containing either MLN or carcass swabs. Before the MLN was cut into small pieces with a sterilized blade, the outside surface was cleaned with alcohol and flamed to avoid cross-contamination. Pre-enriched samples were mixed thoroughly and incubated at 37°C for 24 h. After incubation, 100µl of pre-enriched BPW suspension from each sample was transferred to 9.9 ml of Rappaport-Vassilliadis (RV) broth (Difco, Becton Dickinson) and incubated at 42°C for 24h. A loopful (10µl) of enriched RV suspension was streaked on to a xylose lactose tergitol (XLT4) selective agar plate (Difco, Becton Dickinson) and incubated at 37°C for 24 h. To determine the phenotypic and genotypic diversity of *Salmonella* within a positive sample we selected three black-colored colonies from XLT4 and characterized them biochemically by stabbing into triple sugar iron (TSI) and urea agar slants (Difco, Becton Dickinson). Biochemical testing was interpreted from the TSI and urea agar slants; colonies with a positive TSI and negative urea tests were confirmed as *Salmonella* isolates. Further confirmation of *Salmonella* was performed by PCR amplification of a targeted *Salmonella*-specific invasive (*invA*) gene (23). The confirmed

Salmonella isolates were appropriately labeled and stored in Brucella broth (Difco, Becton Dickinson) at -80°C for further characterization.

2.3.5 *Salmonella* serotyping

All *Salmonella* isolates (n=1,090) were serotyped using one of three methods. Initially, a multiplex PCR was performed to scan the entire isolate set to identify *Salmonella* Typhimurium using published primers and protocols (24, 25). The template DNA for this multiplex PCR was purified using Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. In the second approach, PFGE fingerprint profiles were generated for a subset of 86 isolates and the serotypes were identified by matching their fingerprint profiles with a database of previously confirmed *Salmonella* serotypes (26-28). The remaining isolates (n=684) were sent to the National Veterinary Services Laboratories (NVSL) for traditional phenotypic serotyping.

2.3.6 Antimicrobial susceptibility testing

All the confirmed *Salmonella* isolates (n=1,090) from pigs and the environment were tested against a panel of 15 antimicrobials by the broth micro dilution method (Trek Diagnostic Systems, Inc., Cleveland, OH). The panel of antimicrobials tested, along with their respective concentration ranges increasing twofold, included: amikacin (AMI; 0.5-64), ampicillin (AMP; 1-32), amoxicillin/clavulanic Acid (AUG; 0.5-32/16), ceftriaxone (AXO; 0.25-64), cefoxitin (FOX; 0.5-32), ceftiofur (TIO; 0.25-8), chloramphenicol (CHL; 2-32), ciprofloxacin (CIP; 0.015-2), gentamicin (GEN; 0.25-16), kanamycin (KAN; 8-64), nalidixic acid (NAL; 0.5-32), sulfisoxazole (FIS; 16-256), streptomycin (STR; 32-64), trimethoprim/sulfamethoxazole (SXT; 0.12/2.38-4/76), and tetracycline (TET; 4-32).

Briefly, 10 µl of bacterial culture (adjusted to a 0.5 McFarland standard) was transferred to 11 ml of Mueller-Hinton broth. Using the Sensititre® semi-automated system (Trek Diagnostic Systems, Inc., Cleveland, OH), 50 µl of Mueller-Hinton broth was distributed to each well in a 96-well Sensititre® CMV1AGNF plate (Trek Diagnostic Systems, Inc., Cleveland, OH). The plates were sealed and incubated at 37°C for 24 h. *Escherichia coli* ATCC25922 strain was used for quality control. The MICs were recorded and breakpoints were determined based on Clinical Laboratory Standards Institute (CLSI) recommendations where available (29). National Antimicrobial Resistance Monitoring System (NARMS) consensus breakpoints were used where CLSI breakpoints were indeterminate (5). Those isolates exhibiting resistance to three or more antimicrobials were considered multidrug resistant (MDR).

2.3.7 Pulsed Field Gel Electrophoresis (PFGE) analysis

A total of 340 *Salmonella* isolates representing different sampling stages, types of samples, serotypes and AR profiles (Figure. 4) from pigs (ABF, 36; conventional, 100) and environment (ABF, 32; conventional, 172) were genotyped by PFGE using the CDC's PulseNet protocol (30). Briefly, *Salmonella* isolates were grown overnight on LB agar plates. The culture cells were added to cell suspension buffer (CSB) and the concentration was adjusted between optical densities (OD) of 0.48 - 0.52 using a Dade Microscan turbidity meter. The OD adjusted bacterial cell suspension (400 µl) was lysed using proteinase K (20mg/ml) and intact genomic DNA was digested with 50U of *Xba*I (Roche, USA) restriction enzyme in agarose-embedded plugs. The restriction fragments were separated by electrophoresis in 0.5X TBE buffer, 1% ultra pure agarose (Seakem Gold Agarose, Lonza,

Maine, USA) for 18h at 14ºC in a PFGE (CHEFF DR III, BioRad, USA) using pulsed times of 2.2 to 63.8s. The *Xba*I digested *S.* Braenderup H9812 strain was used as the reference DNA marker. Gels were stained with ethidium bromide (10 mg/ml) for 30 min in 400ml of reagent grade water, followed by two washings with nanopure water and photographed under UV light. The PFGE images were analyzed by BioNumerics software version 6.1(Applied Maths, Belgium). Clonal relationships among these isolates were determined using the unweighted-pair group method using average linkages (UPGMA), with band position tolerance and optimization of 1.5% each.

2.3.8 Statistical Analysis

Statistical analysis was carried out using STATA version 12.1 (Stata Corp, College Station, Texas). Descriptive analysis comparing farm types (ABF versus conventional) consisting of unique longitudinal cohorts (which were nested within each farm type) was carried out before pursuing multivariable analyses. Each farm type, along with the stage of production and type of sample collected for isolation of *Salmonella* was also considered for descriptive analysis before forcing these variables for multivariable modeling. Contingency table analyses without adjustments for clustering by cohort/farm type were carried out using likelihood ratio (LR) χ^2 test statistics for each of the variable/s types and used to examine their association with *Salmonella* prevalence. The LR χ^2 test for *Salmonella* prevalence was also carried out for the source of sampling and stages of production. Separate multivariable analyses for pigs versus their environment were carried out using the logistic regression procedure (XTLOGIT) with either random effects (RE) or generalized estimating equation (GEE) models. The XTLOGIT procedure was used instead of XTMELOGIT (multi-level

hierarchical logistic regression) because of problems achieving convergence in XTMELOGIT given the high numbers of zero cells in the ABF farm type. The main effects of farm type, stage of production, and sample type along with their 2-way and 3-way interaction terms were tested. The final full factorial RE or GEE model was constructed for both main effects and their interaction terms. The final significant model (all variable sets P < 0.05) was selected based on the associations of these variables and their interaction terms with the prevalence of *Salmonella*. The same procedure was repeated by forcing cohorts (farm types) for robust variance estimation and compared. From the final model marginal predictions were obtained for the proportion of positive *Salmonella* and these were estimated with 95% confidence intervals. The marginal means were plotted using final predictions from the full factorial RE or GEE models for: 1) *Salmonella* prevalence among pigs by farm type and different stages of production, 2) *Salmonella* prevalence in environmental samples by farm type and different stages of production, and 3) *Salmonella* prevalence in environmental samples by farm type and different stages of production accounting for sample type differences.

2.4 Results

2.4.1 *Salmonella* prevalence in pigs and the environment at farms

A total of 1,090 *Salmonella* isolates were isolated from the all samples collected in the study population. The overall proportion of samples that were positive for *Salmonella* was higher in the conventional production system, both in pigs (66/1,650, 4%) and the environment (156/1,325, 11.7%) when compared to ABF pigs (2/1,239, 0.16%) and the

environment (5/797, 0.6%). The multivariable analysis using logistic regression generated the final significant model (all variables $P < 0.05$), which was selected based on the associations of these variables and their interaction terms with the estimated prevalence of *Salmonella* and plotted along with 95% confidence intervals (Figure 2.1-2.3). The breakdown of *Salmonella* isolated from pigs and the environment samples by farm, type of farm and stage of production are highlighted in Table 2.1 and Table 2.2, respectively. Overall there were statistically significant differences ($P < 0.05$) between the proportion of samples positive for *Salmonella* in ABF (2/1,239, 0.16%) and conventional (66/1,650, 4%) pigs at the following different sampling stages: farrowing (ABF, 0% ; conventional, 6.7%), nursery 1 (ABF, 0.7% ; conventional, 8.8% ,), nursery 2 (ABF, 0.2%; conventional, 7.2%), finishing 1 (ABF, 0.2%; conventional, 6.7%), finishing 2 (ABF, 0.5% ; conventional, 7.7%) (Figure 2.1).

The overall *Salmonella* prevalence in the environmental samples on conventional farms (156/1,325, 11.7%) was higher than in the ABF farms (5/797, 0.62%). At both farrowing and nursery 1 stages, all the environmental samples from the ABF production system were negative for *Salmonella* (Figure 2.2). Overall, the marginal mean predictions for *Salmonella* in the conventional farm environment were significantly higher than in the ABF farm environment. At nursery 1 (ABF, 1.2%; conventional, 13.5%), finishing 1 (ABF, 0.6%; conventional, 12.5%), finishing 2 (ABF, 1.2%; conventional, 11.8%). On the conventional farms, *Salmonella* was successfully recovered from water, soil, feed, floor swabs, lagoons, and truck samples. Among all the environmental samples, the *Salmonella* mean prediction was higher in lagoons when compared to other environmental samples

(Figure 2.3); on the other hand, on ABF farms only water (nursery 1, 4.8%) and feed (finishing 1, 0.6%; finishing 2, 1.2%) samples were positive for *Salmonella* (Figure 2.3).

2.4.2 *Salmonella* prevalence in carcasses and the environment at slaughter

Overall, the proportion of positive samples for *Salmonella* was significantly higher in MLN from conventional carcasses than ABF carcasses ($P= < 0.001$). However, the prevalence of *Salmonella* in post evisceration and post chill carcass swabs was higher in ABF carcasses than conventional carcasses. There was a statistically significant difference between the post-evisceration swabs (P=0.008) of ABF carcasses and conventional carcasses. The marginal prediction for *Salmonella* was highest in MLN (ABF, 11.3%; conventional, 26.3%), post evisceration swabs (ABF, 7.1%; conventional, 2.2%) and post chill swabs (ABF, 2.5%; conventional, 0.39%) (Figure 2.1). *Salmonella* were also isolated from the slaughter environment. Overall, the prevalence of *Salmonella* was highest in the conventional slaughter environment (38.8%) than in ABF (18.6%) environmental samples, with the highest marginal prediction in lairage (ABF, 26.2%; conventional, 46%) followed by conventional truck (30%) samples. On the other hand, no ABF truck samples tested positive for *Salmonella*.

2.4.3 Identification and distribution of *Salmonella* serotypes

Three different methods were used to identify the different serotypes. *S*. Typhimurium (n=320) were identified by using multiplex PCR and other serotypes by fingerprint profile matching and by traditional phenotypic serotyping at NSVL. We identified 24 *Salmonella* serotypes among the ABF and conventional pigs and the environment at farm and slaughter (Table 2.3). The ABF and conventional production

systems had certain unique *Salmonella* serotypes which were unevenly distributed in each of the respective production systems at farm and slaughter. Certain serotypes, including *S.* Anatum, *S.* Infantis, and *S.* Typhimurium, were isolated from both production systems. The predominant *Salmonella* serotypes in the ABF system on the farm were *S.* Anatum (pigs, 60%; environment, 21.4%), *S.* Give (pigs, 40%; environment, 42.8%), and *S.* Typhimurium (pigs, 0; environment, 21.4%). At slaughter, *S.* Anatum (carcass, 10.4%; environment, 28.5%) and *S.* Infantis (carcass, 39.5%; environment, 60.3%) were the predominant serotypes. *S.* Give was identified only in the ABF system at farm and slaughter. At ABF slaughter, we identified specific serotypes which were not found at the farm level such as, *S.* Infantis, *S.* Braenderup, *S.* Derby, *S.* Inverness, *S.* Muenchen, *S.* Newport, and *S.* London (Table 2.3). The conventional system had a greater variety of serotypes at farm and slaughter (Table 2.3). On the farm, the major serotypes identified were *S.* Typhimurium (pigs, 28.5% of isolates; environment, 35% of isolates), *S.* Infantis (pigs, 16.4%; environment, 13.8%), *S.* Anatum (pigs, 15.8%; environment, 12%), and *S.* Rissen (pigs, 3%; environment, 8.8%). The *S.* Rissen serotype was reported for the first time in pigs in the US. At conventional slaughter, the major serotypes were *S.* Typhimurium (carcasses, 37%; environment, 30%) followed by, *S.* Derby (carcasses, 35.5%; environment, 1%), and *S.* Infantis (carcasses, 6.5%; environment, 48.4%).

2.4.4 Antimicrobial Resistance profile of *Salmonella*

The overall MIC distribution and prevalence of AR *Salmonella* isolates from pigs and their environment at different stages on farm and slaughter are represented in Table 2.4. A total of 1,090 *Salmonella* isolates were tested (ABF, n=168; conventional, n=922) against a

panel of 15 antimicrobials. AR was higher in conventional isolates (resistant, 80%; pansusceptible, 20%) when compared to ABF isolates (resistant, 27%; pansusceptible, 73%). Overall, *Salmonella* isolates exhibited a wide spectrum of AR with the highest frequency of resistance to TET (70.6%), followed by FIS (41.6%) and STR (17.3%). In addition, *Salmonella* isolates exhibited resistance to β-lactams including cephalosporins with highest frequency of resistance to AMP (12.1%), FOX (4.4%), AXO and TIO (4% each). All the isolates from both production systems were susceptible to AMI and CIP. A 'squashtogram' was generated both to illustrate and compare resistance and MIC distribution of *Salmonella* isolates from pigs and the environment in the conventional production system (Table 2.5). *Salmonella* isolates from pigs and the environment exhibited similar AR profiles and MIC distributions for predominant antimicrobials with the exception of TET. Most of the environmental isolates which were resistant to TET had MIC of either 16 µg/mL (0.4%) or 32 µg/mL (78%); on the other hand, the isolates from pigs had MIC of 32 µg/mL (1%) and >32 µg/mL (79%). We observed the highest frequency of resistance in conventional isolates to TET (pigs, 80.3%; environment, 78.3%), followed by FIS (pigs, 56%; environment, 43.4%), STR (pigs, 27.7%; environment, 14.5%) and AMP (pigs, 13.9%; environment, 14.1%).

2.4.5 Distribution and association of MDR patterns with *Salmonella* serotypes

We observed a higher frequency of MDR isolates from the conventional system at various stages of production. Conventional isolates had different MDR patterns (27.5 %, 254/922) associated with various serotypes. The most common MDR patterns, associated serotypes and distribution are presented in Table 2.6. The FIS STR TET (n=72) was the

predominant MDR pattern we found on farm (pigs, 3%; environment, 21%) and slaughter (carcasses, 75%; environment, 1%) associated with the serotype *S*. Derby. Two major MDR patterns were associated with *S.* Anatum, namely: AMP AUG AXO FOX TIO TET (n=25), which was found only at the farm level (pigs, 44%; environment, 56%), and AMP AUG AXO FOX TIO (n=5), which was identified only with farm (40%) and slaughter (60%) environmental isolates. *S.* Typhimurium was associated with five major MDR patterns, with the most common MDR pattern, AMP CHL FIS STR TET (n=41), a penta-resistant pattern common to *S*. Typhimurium DT104, found at both farm (pig, 12%; environment, 41%) and slaughter (carcasses, 39%; environment, 7%). The FIS SXT TET (n=25) was observed in farm and slaughter environments (32%) and carcasses (36%). FIS STR TET (n=18) was found only at slaughter in carcasses (61%) and environment (39%). *Salmonella* serotypes *S.* Anatum and *S.* Typhimurium with MDR patterns highlighting β- lactams including cephalosporins (AMP AUG AXO FOX TIO TET) were found only at farm level. *S.* Heidelberg had a specific MDR pattern (KAN STR TET) that we found only in the environment (100%). In the ABF system, we found only one isolate with an MDR pattern (AMP CHL FIS STR TET) associated with *S.* Typhimurium isolated from a carcass swab at slaughter.

2.4.6 Pulsed Field Gel Electrophoresis (PFGE)

Salmonella isolates (n=340) from pigs and the environment were genotyped by PFGE. Restriction analyses by *Xba*I produced on average 10-16 bands and distributed the 340 isolates into 58 major clusters consisting of isolates with similar PFGE profiles, and another 53 unique PFGE patterns represented by a single isolate each (Figure 2.6). Two

separate dendrograms were created representing genotypic similarity within the same flow at different stages of production of the two distinct production systems (Figure 2.4 and 2.5). *S.* Infantis isolated from lairage and carcass swabs originating from ABF pigs of two cohorts (A2 and A3) had 100% similar fingerprint profiles (Figure 2.4). Within the conventional production system, we found 100% genotypic similarity among *S.* Rissen isolated from pig fecal, MLN, environmental samples including feed, water, floor swab and lagoon at nursery 1, nursery 2, finishing 2 and slaughter representing the same flow (C3) (Figure 2.5). Identical fingerprint patterns were detected (cluster 14; Figure 2.6) among *S.* Infantis isolates from pig and environmental samples of the same flow (C6) at different stages including farrowing (isolate ID: S548, 551, 575, 581and 591), nursery 1 (isolate ID: S783 and 789), finishing 1 (isolate ID: S963 and 984) and slaughter (isolate ID: S1184 and 1195). Furthermore, we found 100% genotypic similarity among *S.* Infantis (FIS TET pattern) isolated from the conventional production system at farrowing, nursery 1, finishing 1 and slaughter including slaughter truck samples (cluster 14; Figure 2.6). All of the genotypically similar pansusceptible *S.* Infantis isolates at slaughter were grouped in respective clusters (clusters 17, 18, 19, and 20; Figure 2.6). The fingerprint profile of *S*. Anatum isolated from the ABF and conventional production systems at different stages of production and sample type were grouped into four major clusters (clusters 9, 10, 11 and 12; Figure 2.6). Even though the ABF and conventional slaughter facilities were different, we identified a fingerprint profile (cluster 10; Figure 2.6) associated with *S*. Anatum highlighting 100% genotypic similarity between the ABF and conventional production system, which includes isolates from pigs and environment samples at farm and slaughter.

2.5 Discussion

This longitudinal study was conducted to determine and compare AR *Salmonella* at their phenotypic and genotypic levels, isolated from pigs and the environment of both ABF and conventional production systems at different stages of production from farm to slaughter. The prevalence of *Salmonella* in the conventional pigs was significantly higher than the ABF pigs at both farm and slaughter, which is in contrast to an earlier study that reported a higher prevalence of *Salmonella* in an ABF production system (7). The low prevalence of *Salmonella* in outdoor ABF pigs at the farm in the present study was in accordance with other previous findings (9). We observed an increase in the prevalence of *Salmonella* in the final stages of production (finishing 1&2) in the conventional system, similar to previous reports of higher prevalence of *Salmonella* among finishing herds. The likely reasons include a previously infected group of pigs at the farm, contaminated transport vehicles or handling and close contact of pigs during transportation (6, 12, 31). Higher prevalence of *Salmonella* in pigs during the final stages of production is of greater concern from a public health and food safety perspective. Only a few studies have been conducted to highlight the prevalence of *Salmonella* in the ABF versus the conventional farm environment (6, 7, 9, 32). In the ABF farm environment, *Salmonella* was only detected in three feed and two water samples at nursery 1 and finishing stages, in contrast to the conventional farm where positive environmental samples were widely represented by water, soil, feed, floor swabs, lagoon and truck at different stages of production. The detection of Salmonella in feed samples of ABF and conventional farm A higher number of conventional environmental samples were positive for *Salmonella,* in spite of strict AIAO practices. This highlights the potential role of

the farm environment as a reservoir, which is in accordance with studies highlighting the persistence of *Salmonella* in the farm environment for several months to years (33, 34). Even though the ABF pigs had access to the external environment throughout their production chain, the prevalence of *Salmonella* in pigs was low which may be attributed to the absence/low prevalence of *Salmonella* in the ABF environment.

In our study, we found a higher prevalence of *Salmonella* in both production systems at slaughter in both pigs and the environment when compared to prevalence at the farm. Factors contributing to the increased prevalence of *Salmonella* at slaughter likely include cross contamination at peri-harvest stage by trucks involved in transfer to the slaughtering facilities, stress experienced by the pigs during transport, cross contamination at lairage and at post-harvest stages (16, 17, 20). In addition, a previous study highlighted that contaminated feed at the end stage of production have significant role in dissemination of *Salmonella* (19). We detected clear evidence of cross contamination in our study as shown in clusters 7, 8, 14, 17, 18 and 26 (Figure 2.4). The MLN samples from both production systems had a higher prevalence of *Salmonella* when compared to fecal samples at the farm, which is in accordance with previous reports suggesting occurrence of *Salmonella* in the gastrointestinal (GI) tract and lymphatic tissue in carrier pigs (6, 14, 17). Even though the MLN and gut contents are not used for consumption, occurrence of *Salmonella* in the MLN may act as a reservoir in contaminating carcasses during the post-evisceration stage. We also isolated *Salmonella* from the post-evisceration carcasses, which were cleansed with water before they were stored in the chilling facility. This indicates possible cross contamination during the evisceration process along the slaughter chain. The ABF slaughter facility had

overnight chilling of the carcass, whereas the conventional slaughter facility had blast chilling. Blast chilling is preferred to overnight cooling because it improves the meat shelf life and tenderness and it prevents the growth of important foodborne pathogens on the carcass surface (35, 36). Interestingly, we isolated *Salmonella* from both the systems in post-chill swabs irrespective of the chilling facility type. The occurrence of *Salmonella* in post-chill swabs (9) is of critical importance to public health and food safety, as because this sample closely represents the final retail product. In this study, we also isolated *Salmonella* from truck floors, which are used to transport conventional and ABF pigs from farm to slaughter. The most significant contribution to positive samples for *Salmonella* at slaughter was from lairage swabs, where pigs rest for about two hours before they are slaughtered. It takes less than two hours for a particular *Salmonella* serotype to establish in the GI tract of pigs and to be shed in their feces (16, 17). Clusters 7 and 8 (Figure 2.4) highlight similar PFGE fingerprint profiles among *Salmonella* isolates from ABF carcass and lairage swabs. However, the detection of *Salmonella* in ABF carcass samples, despite the absence of *Salmonella* at the farm and in transport trucks, clearly highlights the role of lairage and cross-contamination of the carcasses during processing.

Previous studies from Denmark reported various serotypes from organic and outdoor pig farming, including: *S*. Anatum, *S*. Agona, *S*. Derby, *S*. Typhimurium and *S*. Newport, also observed in our ABF isolates (32, 37). However, in the ABF system at the farm and slaughter, we identified the *S*. Give serotype which is most commonly associated with cattle (38). The likely reason for this serotype was the presence of other animals, including cattle, on the same premises as the pigs in the ABF farms and perhaps outdoor access to pastures

which might be an ecological niche. In addition, identification of specific serotypes (Table 2.3) in ABF carcass and processing plant environmental samples, which were not detected at the farm level, suggests the role of the slaughter environment as a source for cross-contamination. Identification of serotypes, including: *S*. Agona, *S*. Braenderup, *S*. Deby, *S*. Inverness, *S*. Muenchen and *S*. Newport on ABF carcass which were not found at farm and slaughter environment (Table 2.3), attributes to the slaughter robots including, carcass splitter and other instruments used for processing the carcass as a possible source of contamination during production chain as described (39). We observed some common serotypes in the ABF and conventional production systems including *S*. Anatum, *S*. Infantis, and *S*. Typhimurium. This is in accordance with a Centers for Disease Control and Prevention (CDC) report of the top four predominant serotypes in swine (40). In the conventional production system, we identified for the first time *S*. Rissen in pigs and the environment. *S*. Rissen is one of the top ten serotype most commonly isolated from pigs since 2004 in Europe (41) and most common non-human serotype in Asian countries (42). According to the CDC annual report, *S*. Rissen was isolated from humans (< 20 isolates per year) from 1999 to 2007 and there were no reports of its occurrence in food animals in the US (41, 43). This serotype is uncommon in the US; it was reported to have entered the US in late 2008 and early 2009 through imported white pepper, resulting in a human outbreak in northern California and Nevada (44). We identified this serotype in our samples collected in late 2009.

Salmonella isolates from conventional production had higher AR (80%) than isolates from ABF production (27%). The use of antimicrobials in the conventional system for treatment and growth purposes likely results in a higher prevalence of *Salmonella* as

previously reported (7, 45). Overall, *Salmonella* isolates of either production system or production stage had the highest frequency of resistance against TET (71%), followed by FIS (42%) and STR (17%). In addition, isolates exhibited resistance to β-lactams, including third generation cephalosporins. These results are in agreement with previous reports (6, 7, 46). MIC distribution was similar for all the antimicrobials tested except TET, which was highest in *Salmonella* isolates of pig origin (MIC > 32 µg/mL; resistance: 80%) when compared to environmental isolates in the conventional system. The possible reasons may be use of tetracyclines as growth promoters administered in feed of growing pigs in our study, which has been reported extensively in the swine industry (47, 48). We detected a higher frequency of MDR isolates in the conventional system (27%) but only a single MDR isolate from the MLN of an ABF carcass exhibiting a penta-resistant pattern of AMP CHL FIS STR TET associated with *S.* Typhimurium. This result is in contrast with a previous report of higher MDR prevalence in an ABF system (7). In the conventional system, *S.* Typhimurium was broadly associated with the common MDR pattern of AMP CHL FIS STR TET at farm and slaughter as previously reported (6, 49). This penta-resistant pattern is common to the *S.* Typhimurium phage type DT104 (50). Identification of this phage type, both at farm and slaughter, is of significant public health concern because this phage type is commonly associated with human foodborne outbreaks worldwide (50, 51). Another important MDR pattern with β-lactams, including third generation cephalosporins (AXO TIO), was associated with *S*. Typhimurium and *S*. Anatum only in conventional pigs and the environment at farm level, as previously reported (6). Emergence of these MDR patterns resistant to β-lactams is

of concern because β-lactams (third generation cephalosporins) are extensively used to treat human clinical *Salmonella* infections (52).

PFGE was used to genotype a representative subset of *Salmonella* isolates from pigs and the environment. PFGE is considered the gold standard test to determine the source of *Salmonella* in epidemiological studies (12, 14). Therefore, we used this genotyping method to determine whether a similar *Salmonella* genotype is disseminated from farm to slaughter along the production chain. Based on similar fingerprint profiles, *Salmonella* isolates in our study were grouped in 58 major clusters. Clustering was consistent with serotypes and resistance patterns as reported by a previous study. In addition, we observed fingerprint profile diversity among the same *Salmonella* serotypes representing different clusters as previously reported (19, 28, 33). Within the conventional production system, 100% genotypic similarity was observed among *S*. Rissen serotype isolates from pig fecal and environmental samples at different stages of production at farm and slaughter from a single cohort (C3). This result highlights the dissemination of relatively new *S.* Rissen serotype in pigs all along the production chain in the US. It was evident that specific serotypes, including *S*. Anatum, *S*. Infantis, *S.* Tyhimurium, *S*. Ouakam, *S*. Give and *S*. Ohio, were able to persist in the pigs and environment at different stages of production based on phenotypic and genotypic evidence (Table 2.3; Figure. 2.6 clusters 7, 10, 14, 31, 37 and 42). The identification of genotypically identical *S.* Infantis from the slaughter environment and carcass samples from the ABF system, which were not observed at the farm level, highlights the importance of the slaughter environment from a food safety perspective. In an epidemiological study it is difficult to determine the exact mechanism and direction of

pathogen transmission between pigs and the environment. However, detection of the same genotype among pigs and environment within distinct production systems clearly suggests the exchange of *Salmonella* strains.

2.6 Conclusions

To summarize, this study demonstrates the presence of AR *Salmonella* in ABF and conventional production systems at farm, slaughter and the environment though at much lower levels in ABF than in conventional systems. The phenotypic and genotypic fingerprint profile results underscore the potential role played by the environment in the persistence and dissemination of transmission of AR *Salmonella* in the two production systems. We detected MDR isolates throughout all the production stages and the environment in the conventional system, which uses antimicrobials for prophylaxis and growth purposes. The detection of AR *Salmonella* in ABF pigs and their environment in the absence of selection pressure is a concern. At the phenotypic level, *Salmonella* isolates from the lairage floor, carcass and MLN had similar resistance patterns and serotypes, which were not detected at the farm level. This highlights the importance of the farm and slaughter environment as separate but important reservoirs and as a crucial link to determining the dissemination of AR *Salmonella* among pigs. Future research should focus on environmental factors to develop a better understanding of the molecular epidemiology of this pathogen in the swine production environment and to reduce the burden of AR *Salmonella* on public health.

2.7 Acknowledgements

We thank the swine producers and swine processing plants for providing us access to their facilities. This work was funded by a U.S. Department of Agriculture National Research Initiative grant (2008-529245), a U.S. Department of Agriculture National Integrated Food Safety Initiative grant (2008-529461), the National Pork Board grant (NPB project # 12-001), and a North Carolina State University Individual Faculty Research and Professional Development Award to Dr. Siddhartha Thakur.

2.8 References

1. Lynne AM, Dorsey LL, David DE, Foley SL. 2009. Characterisation of antibiotic resistance in host-adapted *Salmonella enterica*. Int. J. Antimicrob. Agents. 34:169-172.
2. Scallan E, Hoekstra R, Angulo F, Tauxe R, Widdowson M, Roy S, Jones J, Griffin P. 2011. Foodborne illness acquired in the United States-major pathogens. Emerg. Infect. Dis. 17:7-15.
3. Voetsch AC, Van Gilder TJ, Angulo FJ, Farley MM, Shallow S, Marcus R, Cieslak PR, Deneen VC, Tauxe RV, Group EIPFW. 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin. Infect. Dis. 38 Suppl 3:S127-134.
4. Aarestrup FM, Oliver Duran C, Burch DG. 2008. Antimicrobial resistance in swine production. Anim. Health. Res. Rev. 9:135-148.
5. NARMS. 2009. National Antimicrobial Resistance Monitoring System: Executive Report, p. 1-105.
6. Dorr P, Tadesse D, Zewde B, Fry P, Thakur S, Gebreyes W. 2009. Longitudinal study of *Salmonella* dispersion and the role of environmental contamination in commercial swine production systems. Appl. Environ. Microbiol.75:1478-1486.
7. Gebreyes W, Thakur S, Morrow W. 2006. Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant *Salmonella* in antimicrobial-free and conventional pig production. J. Food. Prot. 69:743-748.

8. USDA. September 2009. Organic agriculture: organic market overview. U.S. Department of Agriculture, Economic Research Service, Washington, DC. http://www.ers.usda.gov/topics/natural-resources-environment/organic-agriculture/organic-market-overview.aspx#.Ucw9oCIkA7V

9. Thakur S, Tadesse D, Morrow M, Gebreyes W. 2007. Occurrence of multidrug resistant *Salmonella* in antimicrobial-free (ABF) swine production systems. Vet. Microbiol. 125:362-367.

10. Ray KA, Warnick LD, Mitchell RM, Kaneene JB, Ruegg PL, Wells SJ, Fossler CP, Halbert LW, May K. 2006. Antimicrobial susceptibility of *Salmonella* from organic and conventional dairy farms. J. Dairy. Sci. 89:2038-2050.

11. Davies PR, Bovee FG, Funk JA, Morrow WE, Jones FT, Deen J. 1998. Isolation of *Salmonella* serotypes from feces of pigs raised in a multiple-site production system. J. Am. Vet. Med. Assoc. 212:1925-1929.

12. Rodriguez A, Pangloli P, Richards H, Mount J, Draughon F. 2006. Prevalence of *Salmonella* in diverse environmental farm samples. J. Food. Prot. 69:2576-2580.

13. Barber DA, Bahnson PB, Isaacson R, Jones CJ, Weigel RM. 2002. Distribution of *Salmonella* in swine production ecosystems. J. Food. Prot. 65:1861-1868.

14. Botteldoorn N, Heyndrickx M, Rijpens N, Grijspeerdt K, Herman L. 2003. *Salmonella* on pig carcasses: positive pigs and cross contamination in the slaughterhouse. J. Appl.Microbiol. 95:891-903.

15. De Busser EV, Maes D, Houf K, Dewulf J, Imberechts H, Bertrand S, De Zutter L. 2011. Detection and characterization of *Salmonella* in lairage, on pig carcasses and intestines in five slaughterhouses. Int. J. Food. Microbiol. 145:279-286.

16. Hernández M, Gómez-Laguna J, Luque I, Herrera-León S, Maldonado A, Reguillo L, Astorga RJ. 2013. *Salmonella* prevalence and characterization in a free-range pig processing plant: Tracking in trucks, lairage, slaughter line and quartering. Int. J. Food. Microbiol.162:48-54.

17. Hurd H, Gailey J, McKean J, Rostagno M. 2001. Rapid infection in market-weight swine following exposure to a *Salmonella* Typhimurium-contaminated environment. Am. J. Vet. Res. 62:1194-1197.

18. Botteldoorn N, Herman L, Rijpens N, Heyndrickx M. 2004. Phenotypic and molecular typing of *Salmonella* strains reveals different contamination sources in two commercial pig slaughterhouses. Appl. Environ. Microbiol. 70:5305-5314.

19. Molla B, Sterman A, Mathews J, Artuso-Ponte V, Abley M, Farmer W, Rajala-Schultz P, Morrow W, Gebreyes W. 2010. *Salmonella enterica* in commercial swine feed and subsequent isolation of phenotypically and genotypically related strains from fecal samples. Appl. Environ. Microbiol. 76:7188-7193.

20. Gebreyes W, Davies P, Turkson P, Morrow W, Funk J, Altier C, Thakur S. 2004. Characterization of antimicrobial-resistant phenotypes and genotypes among *Salmonella enterica* recovered from pigs on farms, from transport trucks, and from pigs after slaughter. J. Food. Prot.67:698-705.

21. Owsley F, Rodning S, Floyd J. 2013. Scheduling All-In/All-Out Swine Production. Alabama Cooperative Extension System. http://www.aces.edu/pubs/docs/A/ANR-0847/ANR-0847.pdf, vol. ANR-0847.

22. USDA. 1996. Guidelines for *Escherichia coli* testing for process control verification in cattle and swine slaughter establishments. http://www.fsis.usda.gov/wps/wcm/connect/cebac8d0-954b-4cc2-abcf-205ad4f4ddcd/Guideline_for_Ecoli_Testing_Cattle_Swine_Estab.pdf?MOD=AJPERES

23. Oliveira S, Santos L, Schuch D, Silva A, Salle C, Canal C. 2002. Detection and identification of *Salmonellas* from poultry-related samples by PCR. Vet.Microbiol. 87:25-35.

24. Kim S, Frye J, Hu J, Fedorka-Cray P, Gautom R, Boyle D. 2006. Multiplex PCR-based method for identification of common clinical serotypes of *Salmonella enterica* subsp enterica. J. Clin. Microbiol. 44:3608-3615.

25. Alvarez J, Sota M, Vivanco A, Perales I, Cisterna R, Rementeria A, Garaizar J. 2004. Development of a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples. J. Clin. Microbiol.42:1734-1738.

26. Zou W, Lin WJ, Foley SL, Chen CH, Nayak R, Chen JJ. 2010. Evaluation of pulsed-field gel electrophoresis profiles for identification of *Salmonella* serotypes. J. Clin. Microbiol. 48:3122-3126.

27. Zou W, Lin WJ, Hise KB, Chen HC, Keys C, Chen JJ. 2012. Prediction system for rapid identification of *Salmonella* serotypes based on pulsed-field gel electrophoresis fingerprints. J. Clin. Microbiol. 50:1524-1532.

28. Liebana E, Guns D, Garcia-Migura L, Woodward MJ, Clifton-Hadley FA, Davies RH. 2001. Molecular typing of *Salmonella* serotypes prevalent in animals in England: assessment of methodology. J. Clin. Microbiol. 39:3609-3616.

29. CLSI. 2010. Performance Standards for Antimicrobial Susceptibility Testing;Twentieth Informational Supplement, p. 40-45, vol. 30.

30. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog. Dis. 3:59-67.

31. Rodríguez-Buenfil JC, Alvarez-Fleites M, Segura-Correa JC. 2006. Incidence of salmonellosis and identification of serogroups and serotypes in a pig commercial farm in Yucatan. Rev. Latinoam. Microbiol. 48:10-13.

32. Jensen AN, Dalsgaard A, Stockmarr A, Nielsen EM, Baggesen DL. 2006. Survival and transmission of *Salmonella enterica* serovar Typhimurium in an outdoor organic pig farming environment. Appl. Environ. Microbiol. 72:1833-1842.

33. Sandvang D, Jensen LB, Baggesen DL, Baloda SB. 2000. Persistence of a *Salmonella enterica* serotype Typhimurium clone in Danish pig production units and farmhouse environment studied by pulsed field gel electrophoresis (PFGE). FEMS. Microbiol. Lett. 187:21-25.

34. Baloda SB, Christensen L, Trajcevska S. 2001. Persistence of a *Salmonella enterica* serovar Typhimurium DT12 clone in a piggery and in agricultural soil amended with *Salmonella*-contaminated slurry. Appl. Environ. Microbiol. 67:2859-2862.

35. Nesbakken T, Eckner K, Røtterud OJ. 2008. The effect of blast chilling on occurrence of human pathogenic *Yersinia enterocolitica* compared to *Campylobacter* spp. and numbers of hygienic indicators on pig carcasses. Int. J. Food. Microbiol. 123:130-133.

36. Shackelford SD, King DA, Wheeler TL. 2012. Chilling rate effects on pork loin tenderness in commercial processing plants. J. Anim. Sci. 90:2842-2849.

37. Jensen AN, Lodal J, Baggesen DL. 2004. High diversity of *Salmonella* serotypes found in an experiment with outdoor pigs. Wageningen J. Life. Sci. 52:109-117.

38. Edrington TS, Schultz CL, Bischoff KM, Callaway TR, Looper ML, Genovese KJ, Jung YS, McReynolds JL, Anderson RC, Nisbet DJ. 2004. Antimicrobial resistance and serotype prevalence of *Salmonella* isolated from dairy cattle in the southwestern United States. Microb. Drug. Resist. 10:51-56.

39. van Hoek AH, de Jonge R, van Overbeek WM, Bouw E, Pielaat A, Smid JH, Malorny B, Junker E, Löfström C, Pedersen K, Aarts HJ, Heres L. 2012. A quantitative approach towards a better understanding of the dynamics of *Salmonella* spp. in a pork slaughter-line. Int. J. Food. Microbiol. 153:45-52.

40. CDC. 2006. Salmonella Surveillance: Annual summary 2005. Centers for Disease Control and Prevention, Atlanta, GA.

41. Team Ee. 2013. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2011 has been published. Euro. Surveill. 18:20449.

42. Galanis E, Lo Fo Wong DM, Patrick ME, Binsztein N, Cieslik A, Chalermchikit T, Aidara-Kane A, Ellis A, Angulo FJ, Wegener HC, Salm-Surv WHOG. 2006. Web-based surveillance and global *Salmonella* distribution, 2000-2002. Emerg. Infect. Dis. 12:381-388.

43. CDC. 2009. *Salmonella* annual summary tables. Centers for Disease Control and Prevention, Atlanta, GA.

44. MMWR. 2010. *Salmonella* Montevideo infections associated with salami products made with contaminated imported black and red pepper — United States, July 2009–April 2010, p. 1638-1671, vol. 59.

45. Cromwell GL. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. 13:7-27.

46. White DG, Zhao S, McDermott PF, Ayers S, Friedman S, Sherwood J, Breider-Foley M, Nolan LK. 2003. Characterization of integron mediated antimicrobial resistance in *Salmonella* isolated from diseased swine. Can. J. Vet. Res. 67:39-47.

47. Akwar HT, Poppe C, Wilson J, Reid-Smith RJ, Dyck M, Waddington J, Shang D, McEwen SA. 2008. Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia. Can. J. Vet. Res. 72:202-210.

48. McEwen SA, Fedorka-Cray PJ. 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. 34 Suppl 3:S93-S106.

49. Gebreyes W, Thakur S, Davies P, Funk J, Altier C. 2004. Trends in antimicrobial resistance, phage types and integrons among *Salmonella* serotypes from pigs, 1997-2000. J. Antimicrob. Chemother. 53:997-1003.

50. Helms M, Ethelberg S, Mølbak K, Group DS. 2005. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emerg. Infect. Dis. 11:859-867.

51. Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ. 1998. Emergence of multidrug-resistant *Salmonella enterica* serotype Typhimurium DT104 infections in the United States. N. Engl. J. Med. 338:1333-1338.

52. Bradford PA. 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14:933-951.

Table 2.1 Breakdown of *Salmonella* isolation by farm and farm type.

Farms[a]	Farm Type	Cohorts[b]	Frequency[c] (N)	Positive (%)
ABF 1	ABF	A1	394	5.9
ABF 2	ABF	A2	333	5.1
ABF 3	ABF	A3	341	5.2
ABF 4	ABF	A4	342	5.2
ABF 5	ABF	A5	366	5.6
ABF 6	ABF	A6	344	5.2
ABF 7	ABF	A7	365	5.6
ABF 8	ABF	A8	232	3.5
CONV 1*	Conventional	C1	388	5.9
CONV 2*	Conventional	C2	362	5.5
CONV 3	Conventional	C3	121	1.84
CONV 4	Conventional	C3	171	2.6
CONV 5	Conventional	C3	72	1
CONV 6	Conventional	C4	195	2.9
CONV 7	Conventional	C4	119	1.8
CONV 8	Conventional	C4	67	1
CONV 9	Conventional	C5	69	1.1
CONV 10	Conventional	C5	70	1.1
CONV 11	Conventional	C5	286	4.4
CONV 12	Conventional	C6	69	1.1
CONV 13	Conventional	C6	67	1
CONV 14	Conventional	C6	239	3.6
CONV 15	Conventional	C7	37	1
CONV 16	Conventional	C7	127	1.9
CONV 17	Conventional	C7	215	3.3
CONV 18*	Conventional	C8	210	3.2
CONV 19*	Conventional	C8	185	2.8
CONV 20	Conventional	C9	194	2.9
CONV 21	Conventional	C9	67	1
CONV 22	Conventional	C9	122	1.9
CONV 23*	Conventional	C10	183	2.8
CONV 24*	Conventional	C10	197	3

[a]ABF1-8: Antimicrobial Free farm owned by individual farmers; CONV1-24: conventional farms owned by two different companies.

Table 2.1(continued)

*The total number of conventional farm sampled was 30. The *indicated farms sampled at three different locations, since the name of the farm is same they appeared as single farm. Therefore there are only 24 farms listed in the table.

[b]A1-8: ABF cohorts; C1-10: Conventional cohorts

[c]Frequency-No of samples collected which includes fecal, environmental and slaughter samples

Table 2.2 Breakdown of *Salmonella* isolation by production/processing stage from ABF and conventional production system.

Production Stage[a]	Frequency[b] (N)	Positive (%)
Farrowing	1,112	16.9
Nursery1	1,026	15.6
Nursery2	974	14.8
Finishing1	986	14.9
Finishing2	913	13.8
Slaughter	672	10.21
Post Evis	454	6.9
Post Chill	454	6.7

[a]Farrowing: includes fecal samples from sows and piglets, environmental samples; nursery1 and 2, finishing 1and 2: includes fecal and environmental samples; salughter: includes mesenteric lymphnodes, lairage and truck swabs; Post Evis: post evisceration; Post-Chill: post chilling

[b]Frequency: total number of samples collected from pigs and environment

Table 2.3 The distribution of *Salmonella* serotypes from pigs and the environmental samples at farm and slaughter (n=1,090).

Serotypes Identified	**Farm**				**Slaughter**			
	ABF		**Conventional**		**ABF**		**Conventional**	
	Pigs n=5	Environment n=14	Pigs n=189	Environment n=439	Carcass n=86	Environment n=63	Carcass n=197	Environment n=97
S. Agona	0	0	0	0	1 (1.1)	0	(0.5)	0
S. Anatum	3 (60)	3 (21.4)	30 (15.8)	53 (12)	9 (10.4)	18 (28.5)	(3)	15 (15.4)
S. Braenderup	0	0	0	1 (0.2)	3 (3.4)	0		0
S. Cerro	0	0	12 (6.3)	3 (0.6)	0	0		0
S. Derby	0	0	4 (2.1)	31 (7)	5 (5.8)	0	[illegible] (35.5)	1 (1)
S. Give	2 (40)	6 (42.8)	0	0	3 (3.4)	0		0
S. Heidelberg	0	0	0	17 (3.8)	0	0		0
S. Infantis	0	0	31 (16.4)	61 (13.8)	34 (39.5)	38 (60.3)	[illegible] (6.5)	47 (48.4)
S. Inverness	0	0	0	2 (0.4)	13 (15.1)	0		0
S. Johannesburg	0	0	0	1 (0.2)	0	0	(2.5)	0
S. London	0	0	0	3 (0.6)	0	3 (4.7)		2 (2)

Table 2.3 (continued)

S. Mbandaka	0	0	2 (1)	2 (0.4)	0	0	3 (1.5)	1 (1)
S. Muenchen	0	0	3 (1.5)	0	11 (12.7)	0	0	0
S. Newport	0	0	0	1 (0.2)	3 (3.4)	0	0	0
S. Ohio	0	0	20 (10.5)	24 (5.4)	0	0	6 (3)	1 (1)
S. Ouakam	0	0	23 (12.1)	41 (9.3)	0	0	8 (4)	0
S. Rissen	0	0	6 (3.1)	39 (8.8)	0	0	1 (0.5)	0
S. Rough_O:r:1,5	0	0	0	0	0	1 (1.5)	9 (4.5)	0
S. Schwarzengrund	0	0	0	2 (0.4)	0	0	0	0
S. Senftenberg	0	0	4 (2.1)	2 (0.4)	0	0	1 (0.5)	0
S. Typhimurium	0	3 (21.4)	54 (28.5)	154 (35)	1 (1.1)	3 (4.7)	73 (37)	30 (30.1)
S. Typhimurium Var 5	0	2 (14.2)	0	0	0	0	1 (0.5)	0
6,7, Non motile	0	0	0	2 (0.4)	0	0	0	0
III_44:z4,z32:-	0	0	0	0	3 (3.4)	0	0	0

Table 2.4 The MIC distribution (squashtogram) of *Salmonella* isolated from all swine samples (n=1,090)*

AR[a]		**Distribution of MICs in µg/mL (%)**															
	% R	**0.015**	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
AMI	0.0						0.6	65.2	30.8	2.9	0.4	0.0	0.0	0.0	0.0		
AMP	12.1							78.4	5.0	4.4	0.1	0.0	0.3	11.8			
AUG**	4.1							82.7	3.2	2.0	1.3	6.7	2.9	1.2			
AXO	4.0					95.7	0.1	0.2	0.1	0.0	0.1	0.6	3.1	0.0	0.2		
CHL	5.0								1.3	32.9	58.9	2.0	0.4	4.6			
CIP	0.0	92.2	4.7	0.9	0.3	0.1	1.7	0.1	0.0	0.0							
FIS	41.6											0.8	0.8	53.6	1.9	1.3	41.6
FOX	4.4							1.3	17.3	73.8	2.1	1.2	0.6	3.9			
GEN	0.5					63.5	30.6	4.5	0.1	0.0	0.8	0.1	0.4				
KAN	2.1										97.8	0.0	0.0	0.1	2.1		
NAL	1.9						0.3		21.6	75.1	1.0	0.2	0.2	1.7			
STR	17.3												82.7	4.5	12.8		
SXT**	3.1				94.5	0.3	0.8	1.1	0.2	3.1							
TET	70.6									29.4	0.0	0.0	0.7	69.9			
TIO	3.9				0.1	0.4	11.3	82.5	1.7	0.2	0.0	3.9					

*The whitened areas indicate the range of dilutions tested for each antimicrobial. Shaded areas fall outside the range of tested concentrations. The vertical bars indicate the CLSI or NARMS consensus breakpoints for resistance (R versus I and S combined).

Table 2.4 (continued)

Numbers in the right-side shaded areas indicate the percentage of isolates with undetermined MICs known to be greater than the highest concentrations measured on the microbroth dilution plates.

**The MIC represents for the first antibiotic (of two).

[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

Table 2.5 Comparison of resistance and MIC distribution (squashtogram) for *Salmonella* isolated from the conventional production system at farm and slaughter (pigs n=386; environment n=536)*.

AR[c]	Source	% R	Distribution of MICs in µg/mL (%)															
			0.015	0.03	0.06	0.13	0.25	0.5	1	2	4	8	16	32	64	128	256	512
AMP	Pigs[a]	13.9							76.2	3.9	6	0	0	0.2	13.7			
	Envi[b]	14.1							76.6	5.4	3.5	0.2	0	0.4	13.8			
AXO	Pigs	4.1					50.5	0	0	0.2	0	0	0.2	3.6	0	0.2		
	Envi	5					94.4	0	0	0.2	0	0.2	1.1	3.7	0			
CHL	Pigs	8								0.7	34.9	54.4	1.8	0.7	7.2			
	Envi	4.1								1.1	35	56.9	2.7	1.1	3.9			
FIS	Pigs	55.9											0	0.8	43.2	0	0	79.5
	Envi	43.4											0.7	0.4	55.4	0	0	43.4
FOX	Pigs	4.4						0	0.2	20.7	71	2.5	1	0.5	3.8			
	Envi	5.2						0	1.5	13.2	76.3	2	1.6	0.4	4.8			
STR	Pigs	27.7												72.2	4.4	23.3		
	Envi	14.5												85.4	5.2	9.3		
TET	Pigs	80.3									19.6	0	0	1	79.2			
	Envi	78.3									21.6	0	0.4	78				
TIO	Pigs	4.1				0	0.2	8.5	84.1	2.3	0	0	4.1					
	Envi	4.8				0	0.2	11	82	1.8	0.4	0	4.8					

*The whitened areas indicate the range of dilutions tested for each antimicrobial. Shaded areas fall outside the range of tested concentrations. The vertical bars indicate the CLSI or NARMS consensus breakpoints for resistance (R versus I and S combined).

Table 2.5 (continued)

Numbers in the right-side shaded areas indicate the percentage of isolates with undetermined MICs known to be greater than the highest concentrations measured on the microbroth dilution plates.

[a]Pigs includes isolates from conventional pig fecal (n=189) at the farm and carcass samples (n=197) at slaughter

[b]Environment includes isolates from conventional farm (n=439) and slaughter (n=97) environments

[c]AMI: Amikacin, AMP: Ampicillin, AXO: Ceftriaxone, CHL: Chloramphenicol, FIS: Sulfisoxazole, FOX: Cefoxitin, STR: Streptomycin, TET: Tetracycline, TIO: Ceftiofur.

Table 2.6 Distribution of *Salmonella* serotypes associated with predominant MDR patterns in conventional production system.

		Farm		Slaughter	
Serotypes (n)	**Predominant MDR patterns[a] (n)**	**Pigs (%)**	**Environment (%)**	**Carcasses (%)**	**Environment (%)**
S. Anatum (103)	AMP AUG AXO FOX TIO TET (25)	11 (44)	14 (56)	0	0
	AMP AUG AXO FOX TIO (5)	0	2 (40)	0	3 (60)
S. Typhimurium (311)	AMP AUG AXO FOX TIO TET (7)	3 (43)	4 (57)	0	0
	AMP FIS NAL STR TET (13)	3 (23)	8 (61)	1 (8)	0
	AMP CHL FIS STR TET (41)	5 (12)	17 (41)	16 (39)	3 (7)
	FIS SXT TET (25)	0	8 (32)	9 (36)	8 (32)
	FIS STR TET (18)	0	0	11 (61)	7 (39)
S. Derby (106)	FIS STR TET (72)	2 (3)	15 (21)	54 (75)	1 (1)
S. Heidelberg (17)	KAN STR TET (11)	0	11 (100)	0	0

[a]AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, FIS: Sulfisoxazole, FOX: Cefoxitin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, TET: Tetracycline, TIO: Ceftiofur.

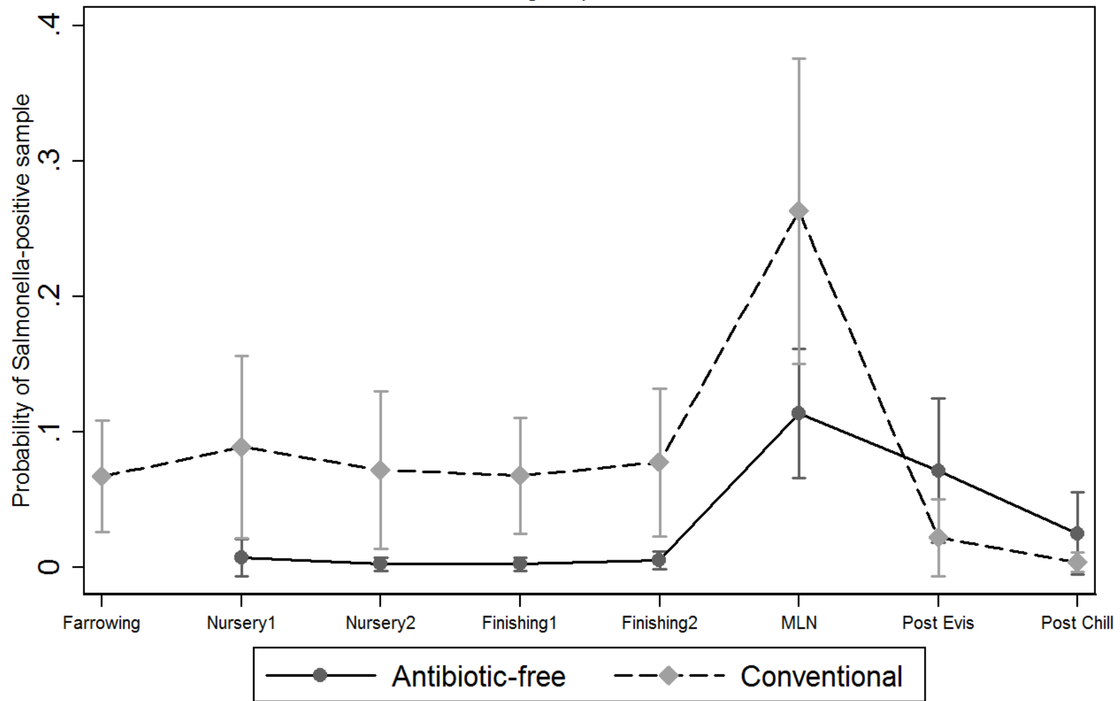


Figure 2.1 *Salmonella* prevalence among pigs at farm and slaughter.

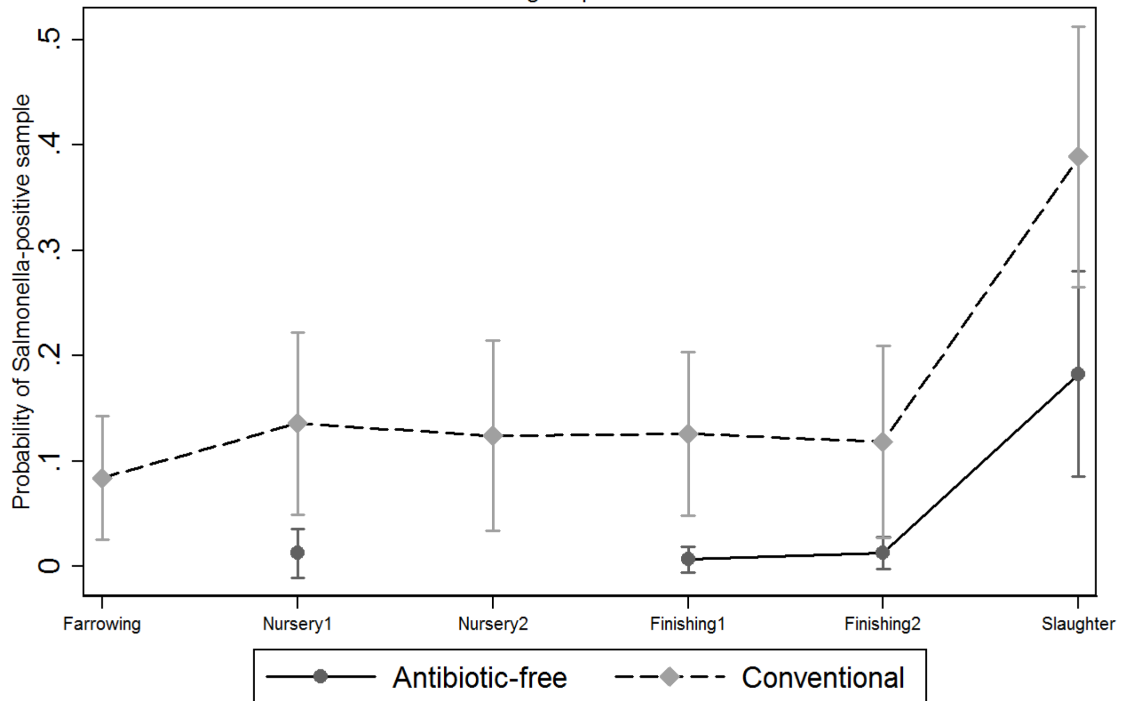


Figure 2.2 *Salmonella* prevalence in the environment at farm and slaughter.

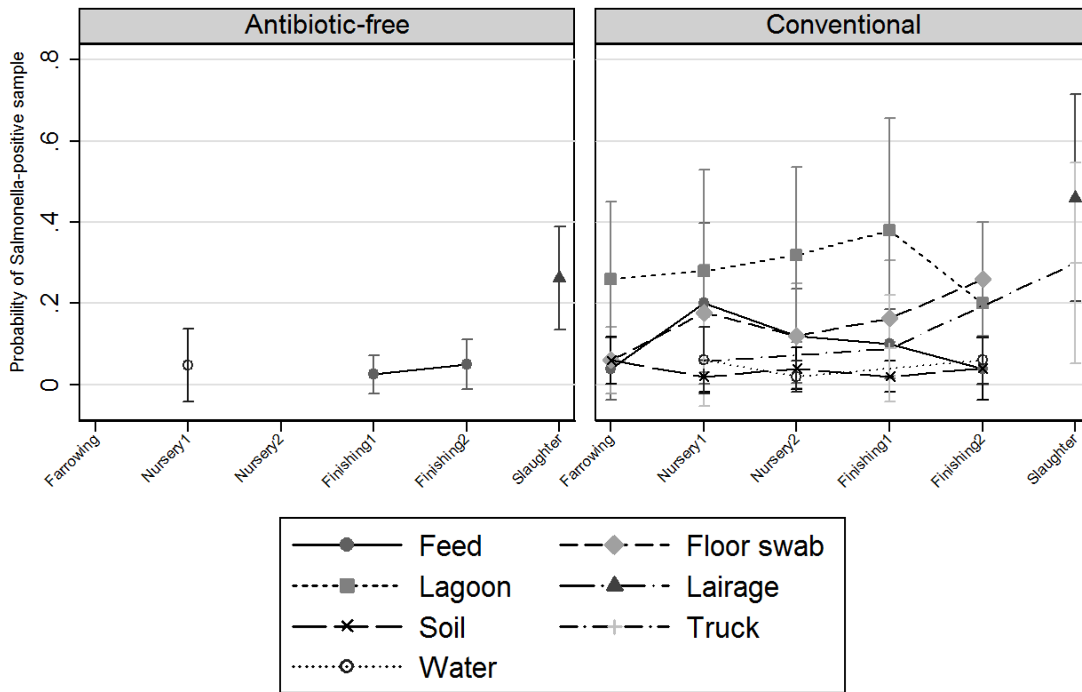


Figure 2.3 *Salmonella* prevalence among environmental samples at farm and slaughter.

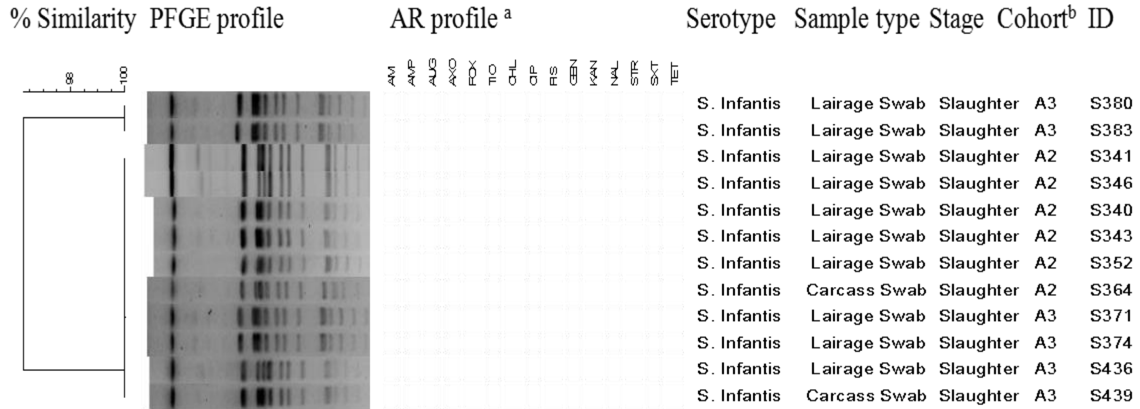


Figure 2.4 Dendrogram showing genotypic similarity among *Salmonella* isolated from ABF systems at various stages of productions.

[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

[b]A2 and A3: ABF cohort

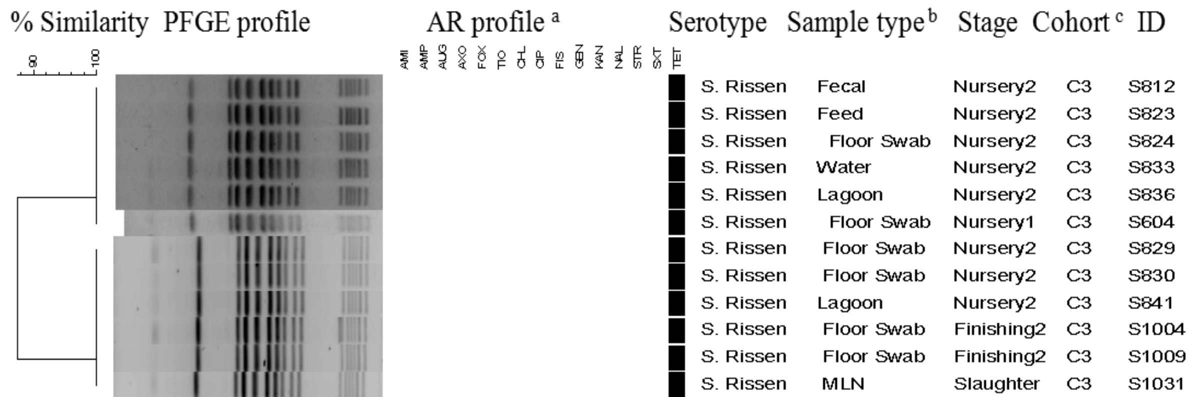


Figure 2.5 Dendrogram showing genotypic similarity among *Salmonella* isolated from conventional system at various stages of productions.

[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

[b]MLN: mesenteric lymphnode

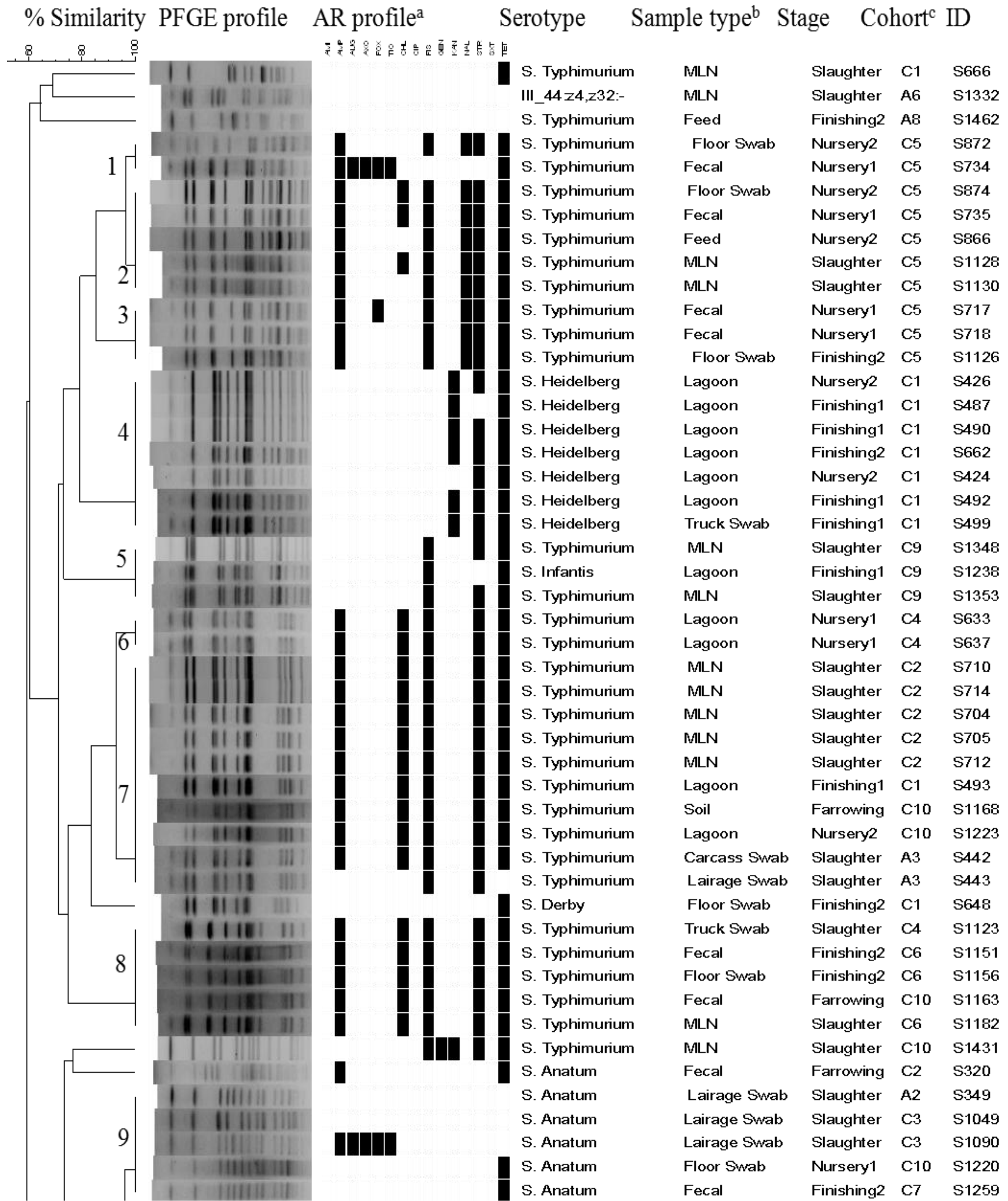
[c]C3: conventional cohort

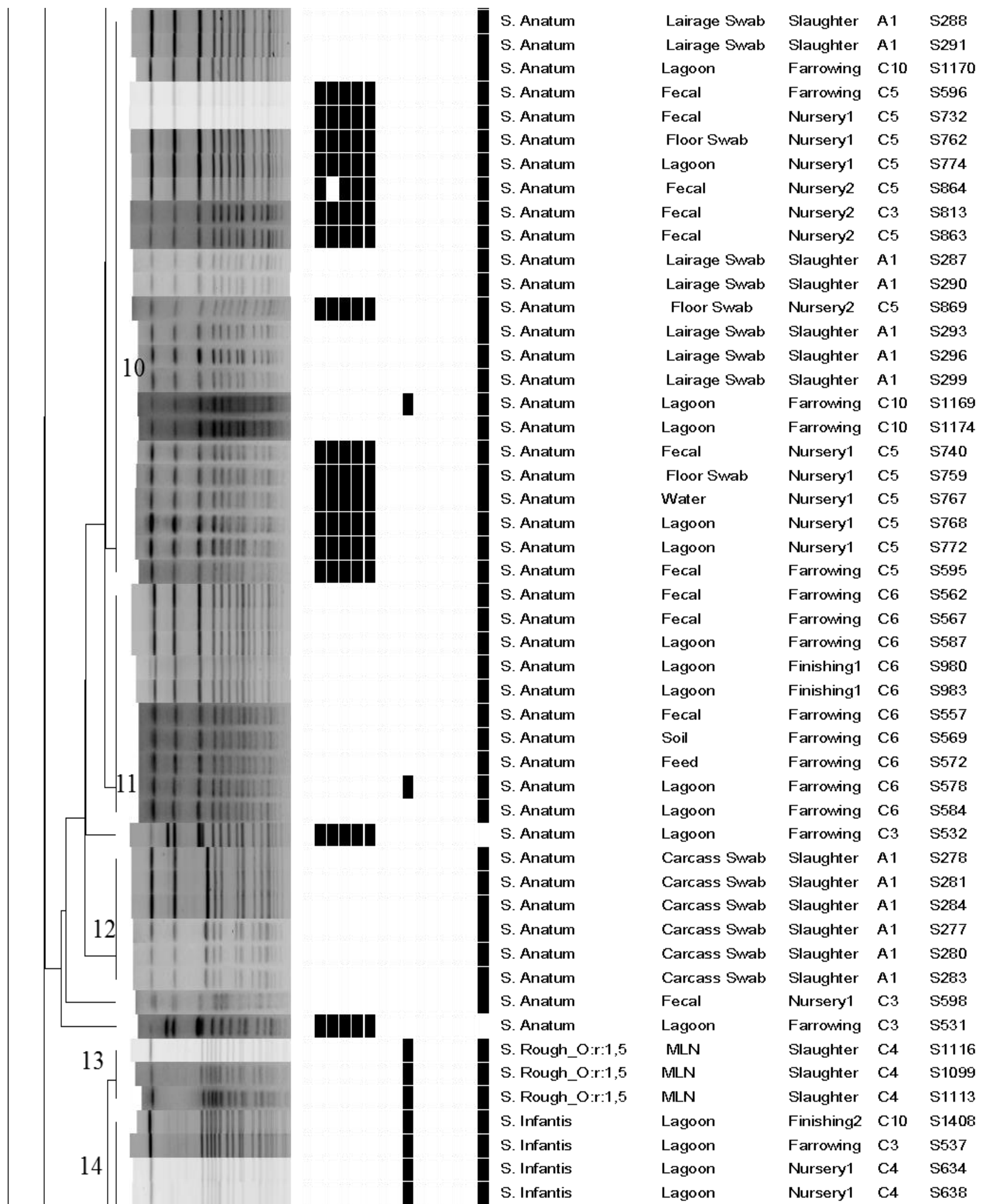
Figure 2.6 Dendrogram showing genotypic similarity among *Salmonella* isolated from conventional and ABF systems at various stages of production (Supplement).

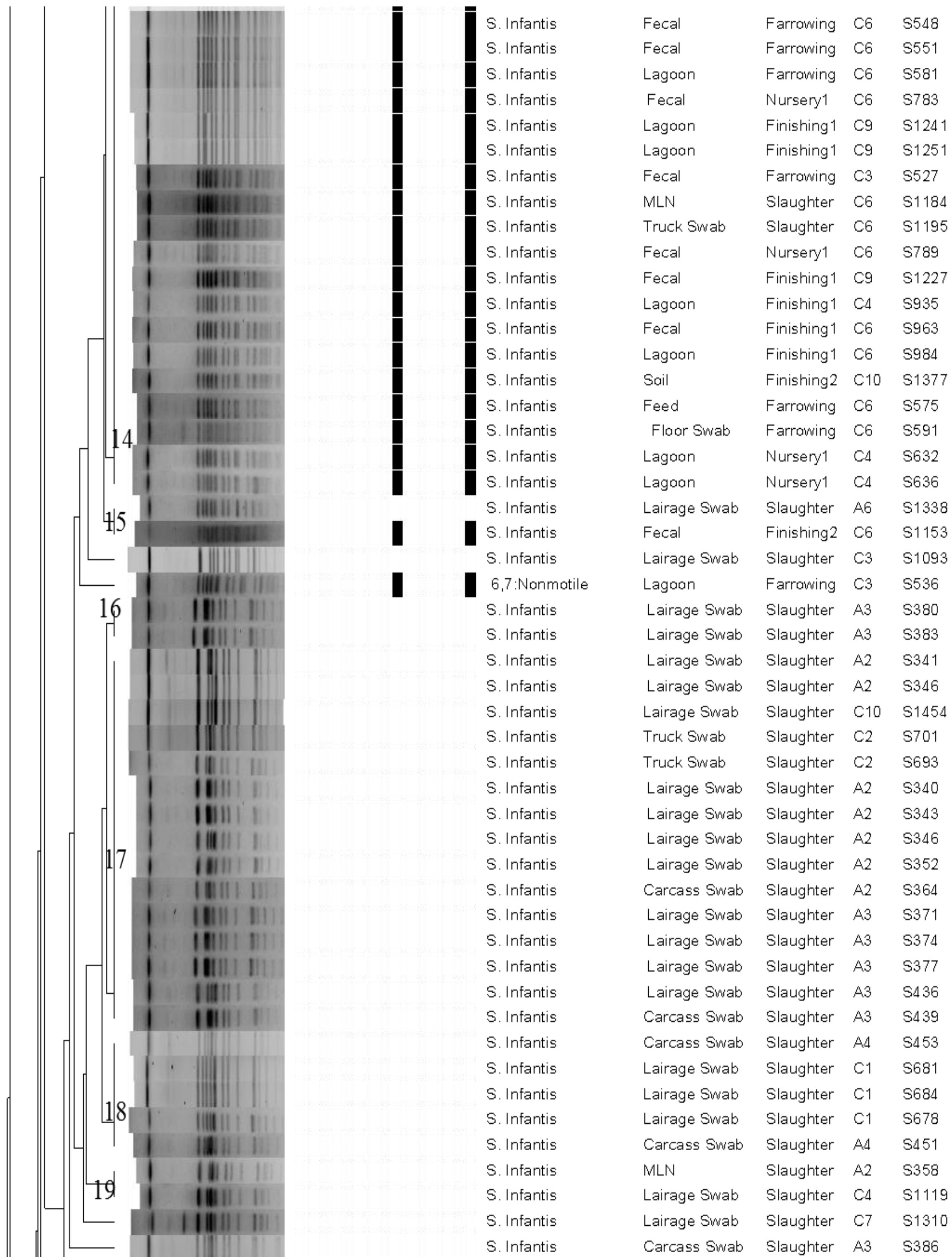
[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

[b]MLN: Mesenteric lymphnode

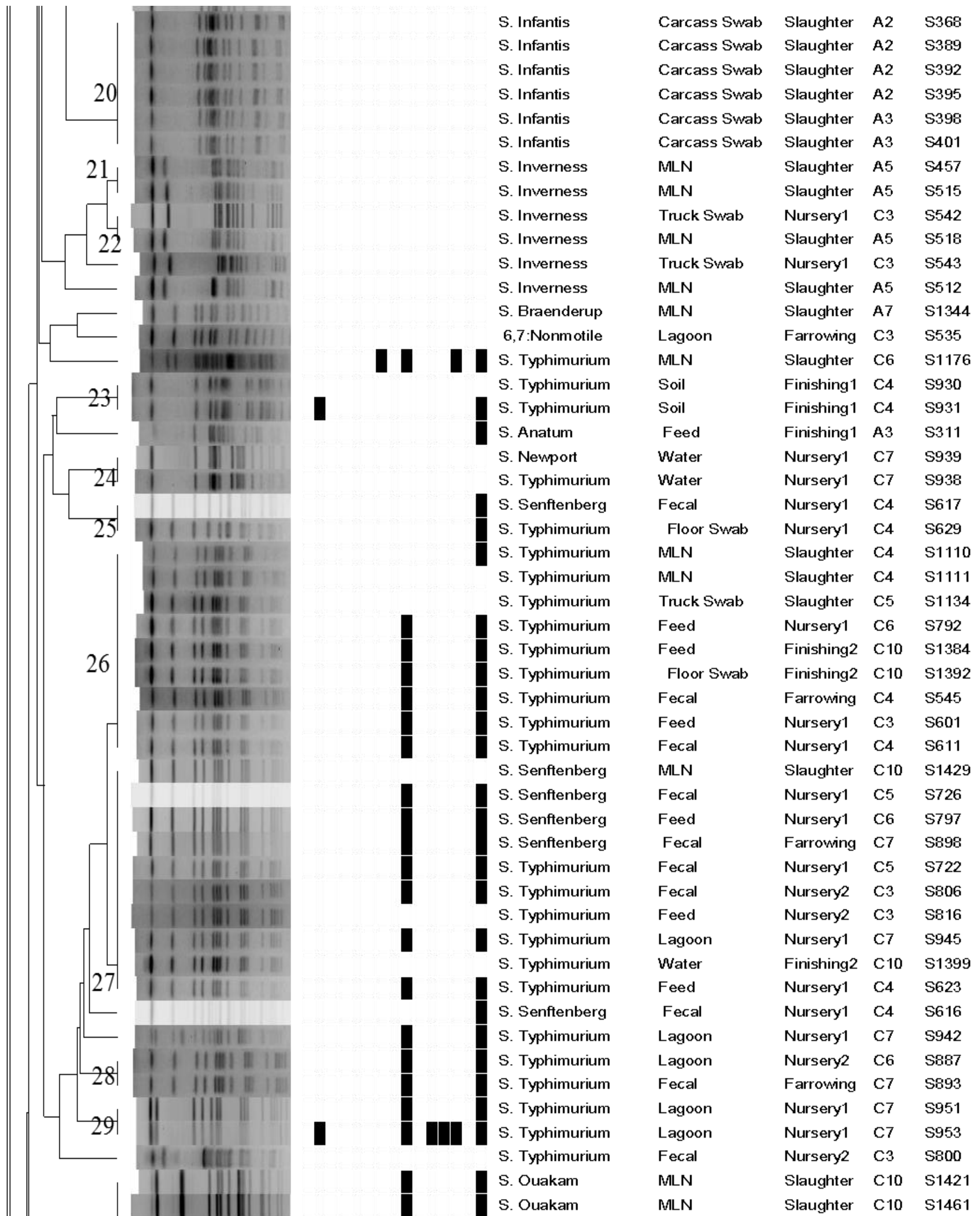
[c]A1-A8: ABF cohorts from 1 to 8; C1-C10: Conventional cohorts

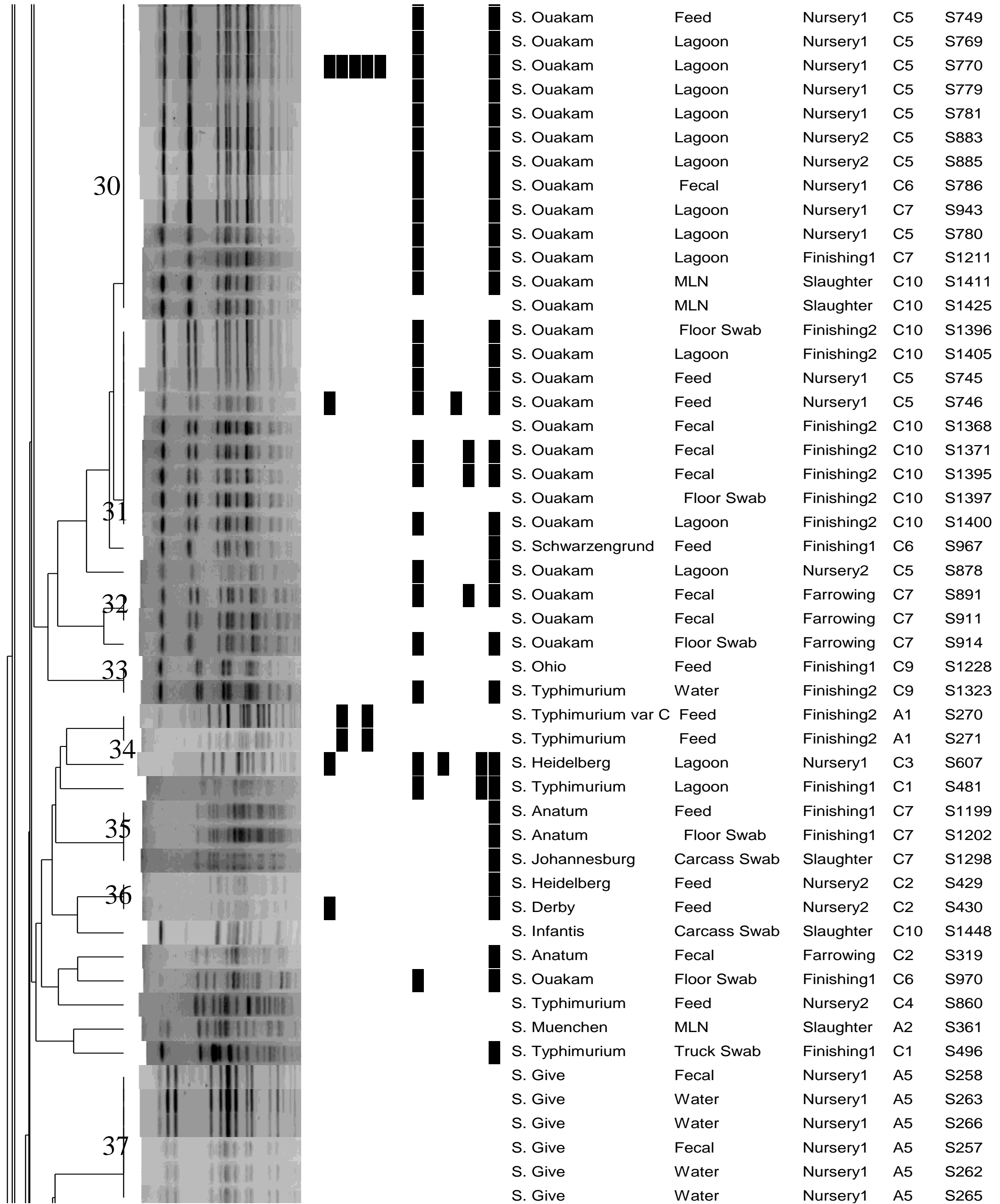

% Similarity
PFGE profile
AR profile[a]
Serotype
Sample type[b]
Stage
Cohort[c]
ID
60
80
100
1
2
3
4
5
6
7
8
9
S. Typhimurium MLN Slaughter C1 S666
III_44:z4,z32:- MLN Slaughter A6 S1332
S. Typhimurium Feed Finishing2 A8 S1462
S. Typhimurium Floor Swab Nursery2 C5 S872
S. Typhimurium Fecal Nursery1 C5 S734
S. Typhimurium Floor Swab Nursery2 C5 S874
S. Typhimurium Fecal Nursery1 C5 S735
S. Typhimurium Feed Nursery2 C5 S866
S. Typhimurium MLN Slaughter C5 S1128
S. Typhimurium MLN Slaughter C5 S1130
S. Typhimurium Fecal Nursery1 C5 S717
S. Typhimurium Fecal Nursery1 C5 S718
S. Typhimurium Floor Swab Finishing2 C5 S1126
S. Heidelberg Lagoon Nursery2 C1 S426
S. Heidelberg Lagoon Finishing1 C1 S487
S. Heidelberg Lagoon Finishing1 C1 S490
S. Heidelberg Lagoon Finishing2 C1 S662
S. Heidelberg Lagoon Nursery2 C1 S424
S. Heidelberg Lagoon Finishing1 C1 S492
S. Heidelberg Truck Swab Finishing1 C1 S499
S. Typhimurium MLN Slaughter C9 S1348
S. Infantis Lagoon Finishing1 C9 S1238
S. Typhimurium MLN Slaughter C9 S1353
S. Typhimurium Lagoon Nursery1 C4 S633
S. Typhimurium Lagoon Nursery1 C4 S637
S. Typhimurium MLN Slaughter C2 S710
S. Typhimurium MLN Slaughter C2 S714
S. Typhimurium MLN Slaughter C2 S704
S. Typhimurium MLN Slaughter C2 S705
S. Typhimurium MLN Slaughter C2 S712
S. Typhimurium Lagoon Finishing1 C1 S493
S. Typhimurium Soil Farrowing C10 S1168
S. Typhimurium Lagoon Nursery2 C10 S1223
S. Typhimurium Carcass Swab Slaughter A3 S442
S. Typhimurium Lairage Swab Slaughter A3 S443
S. Derby Floor Swab Finishing2 C1 S648
S. Typhimurium Truck Swab Slaughter C4 S1123
S. Typhimurium Fecal Finishing2 C6 S1151
S. Typhimurium Floor Swab Finishing2 C6 S1156
S. Typhimurium Fecal Farrowing C10 S1163
S. Typhimurium MLN Slaughter C6 S1182
S. Typhimurium MLN Slaughter C10 S1431
S. Anatum Fecal Farrowing C2 S320
S. Anatum Lairage Swab Slaughter A2 S349
S. Anatum Lairage Swab Slaughter C3 S1049
S. Anatum Lairage Swab Slaughter C3 S1090
S. Anatum Floor Swab Nursery1 C10 S1220
S. Anatum Fecal Finishing2 C7 S1259

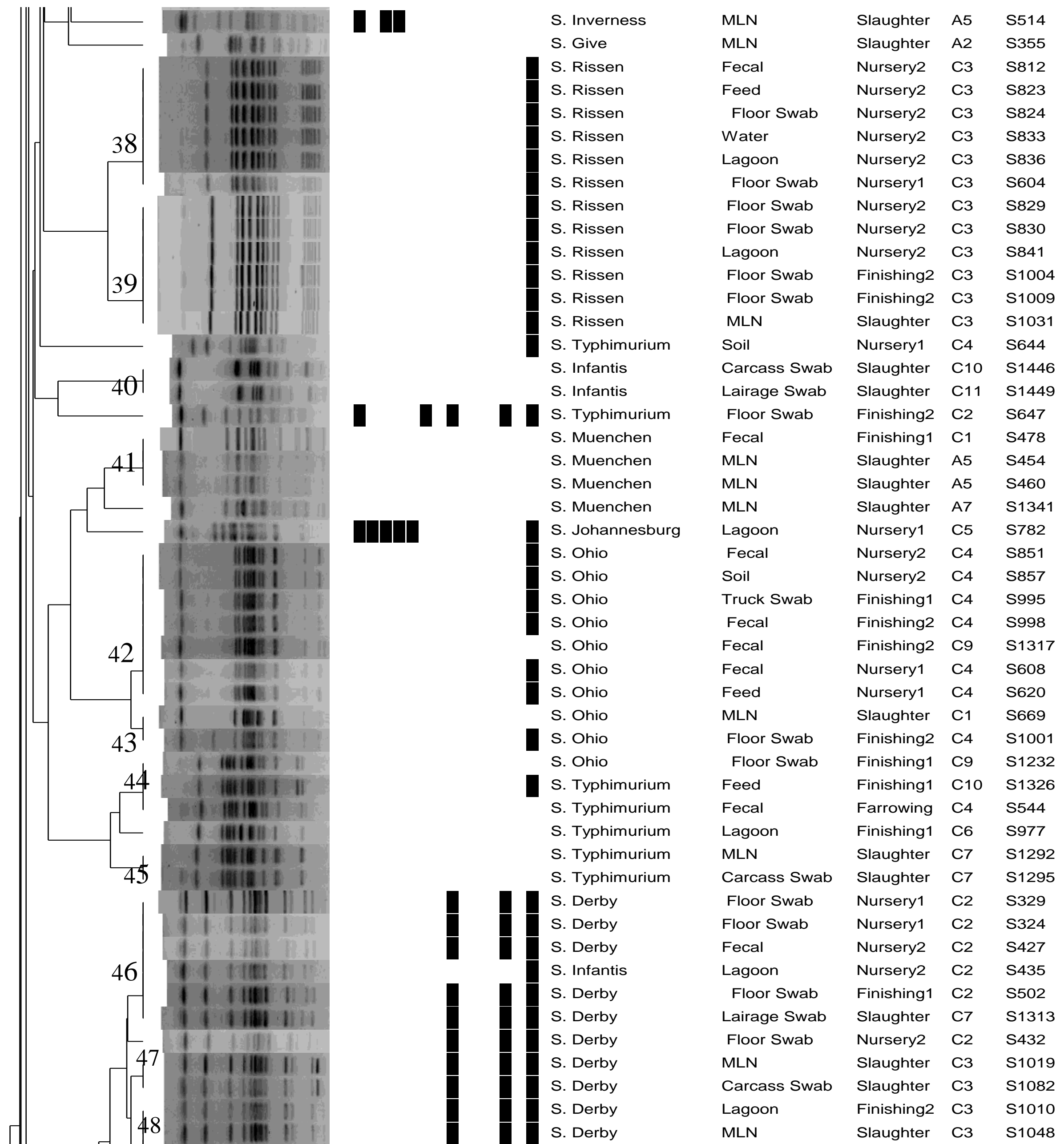

10
11
12
13
14
S. Anatum Lairage Swab Slaughter A1 S288
S. Anatum Lairage Swab Slaughter A1 S291
S. Anatum Lagoon Farrowing C10 S1170
S. Anatum Fecal Farrowing C5 S596
S. Anatum Fecal Nursery1 C5 S732
S. Anatum Floor Swab Nursery1 C5 S762
S. Anatum Lagoon Nursery1 C5 S774
S. Anatum Fecal Nursery2 C5 S864
S. Anatum Fecal Nursery2 C3 S813
S. Anatum Fecal Nursery2 C5 S863
S. Anatum Lairage Swab Slaughter A1 S287
S. Anatum Lairage Swab Slaughter A1 S290
S. Anatum Floor Swab Nursery2 C5 S869
S. Anatum Lairage Swab Slaughter A1 S293
S. Anatum Lairage Swab Slaughter A1 S296
S. Anatum Lairage Swab Slaughter A1 S299
S. Anatum Lagoon Farrowing C10 S1169
S. Anatum Lagoon Farrowing C10 S1174
S. Anatum Fecal Nursery1 C5 S740
S. Anatum Floor Swab Nursery1 C5 S759
S. Anatum Water Nursery1 C5 S767
S. Anatum Lagoon Nursery1 C5 S768
S. Anatum Lagoon Nursery1 C5 S772
S. Anatum Fecal Farrowing C5 S595
S. Anatum Fecal Farrowing C6 S562
S. Anatum Fecal Farrowing C6 S567
S. Anatum Lagoon Farrowing C6 S587
S. Anatum Lagoon Finishing1 C6 S980
S. Anatum Lagoon Finishing1 C6 S983
S. Anatum Fecal Farrowing C6 S557
S. Anatum Soil Farrowing C6 S569
S. Anatum Feed Farrowing C6 S572
S. Anatum Lagoon Farrowing C6 S578
S. Anatum Lagoon Farrowing C6 S584
S. Anatum Lagoon Farrowing C3 S532
S. Anatum Carcass Swab Slaughter A1 S278
S. Anatum Carcass Swab Slaughter A1 S281
S. Anatum Carcass Swab Slaughter A1 S284
S. Anatum Carcass Swab Slaughter A1 S277
S. Anatum Carcass Swab Slaughter A1 S280
S. Anatum Carcass Swab Slaughter A1 S283
S. Anatum Fecal Nursery1 C3 S598
S. Anatum Lagoon Farrowing C3 S531
S. Rough_O:r:1,5 MLN Slaughter C4 S1116
S. Rough_O:r:1,5 MLN Slaughter C4 S1099
S. Rough_O:r:1,5 MLN Slaughter C4 S1113
S. Infantis Lagoon Finishing2 C10 S1408
S. Infantis Lagoon Farrowing C3 S537
S. Infantis Lagoon Nursery1 C4 S634
S. Infantis Lagoon Nursery1 C4 S638



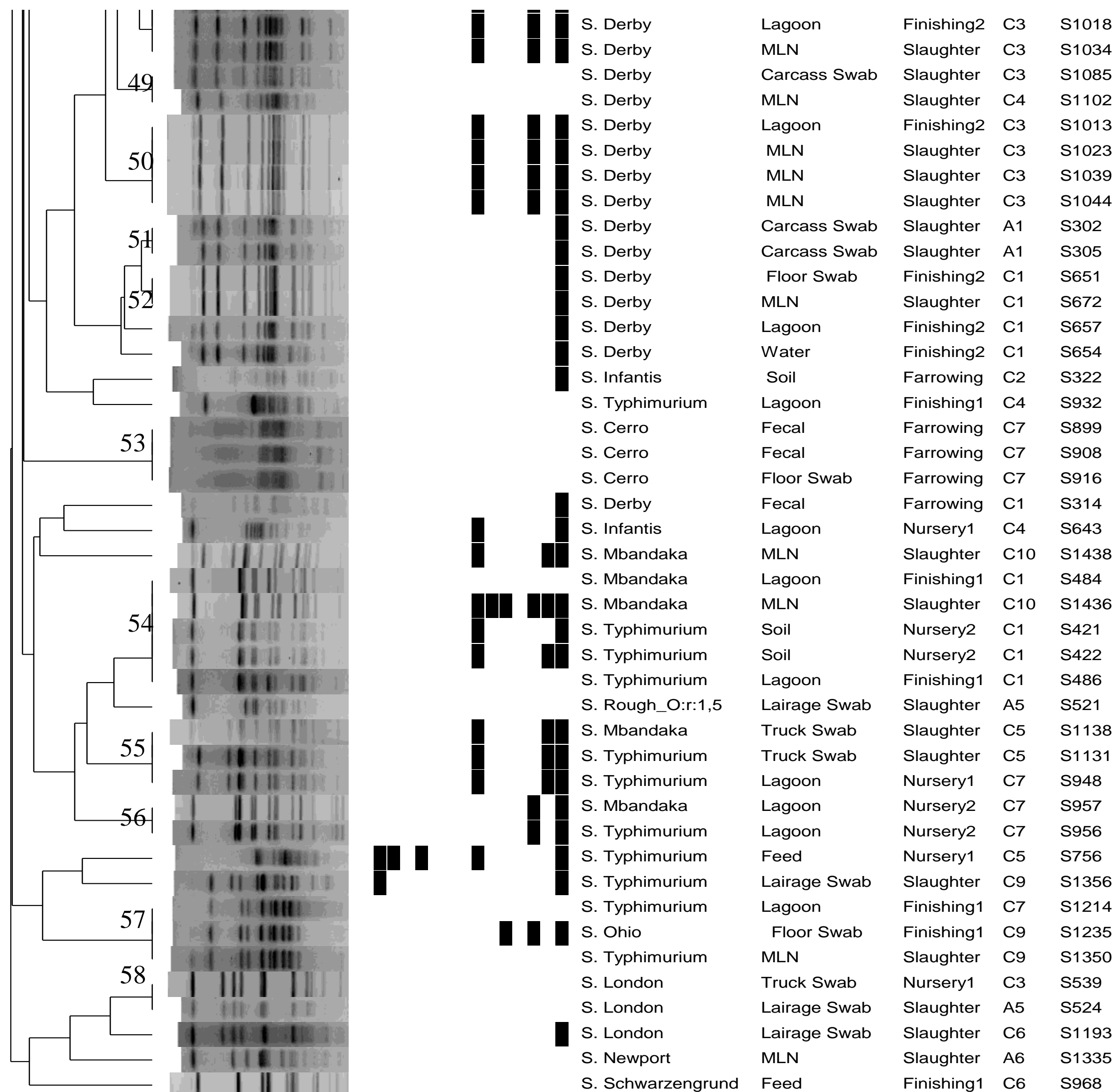
14
15
16
17
18
19
S. Infantis Fecal Farrowing C6 S548
S. Infantis Fecal Farrowing C6 S551
S. Infantis Lagoon Farrowing C6 S581
S. Infantis Fecal Nursery1 C6 S783
S. Infantis Lagoon Finishing1 C9 S1241
S. Infantis Lagoon Finishing1 C9 S1251
S. Infantis Fecal Farrowing C3 S527
S. Infantis MLN Slaughter C6 S1184
S. Infantis Truck Swab Slaughter C6 S1195
S. Infantis Fecal Nursery1 C6 S789
S. Infantis Fecal Finishing1 C9 S1227
S. Infantis Lagoon Finishing1 C4 S935
S. Infantis Fecal Finishing1 C6 S963
S. Infantis Lagoon Finishing1 C6 S984
S. Infantis Soil Finishing2 C10 S1377
S. Infantis Feed Farrowing C6 S575
S. Infantis Floor Swab Farrowing C6 S591
S. Infantis Lagoon Nursery1 C4 S632
S. Infantis Lagoon Nursery1 C4 S636
S. Infantis Lairage Swab Slaughter A6 S1338
S. Infantis Fecal Finishing2 C6 S1153
S. Infantis Lairage Swab Slaughter C3 S1093
6,7:Nonmotile Lagoon Farrowing C3 S536
S. Infantis Lairage Swab Slaughter A3 S380
S. Infantis Lairage Swab Slaughter A3 S383
S. Infantis Lairage Swab Slaughter A2 S341
S. Infantis Lairage Swab Slaughter A2 S346
S. Infantis Lairage Swab Slaughter C10 S1454
S. Infantis Truck Swab Slaughter C2 S701
S. Infantis Truck Swab Slaughter C2 S693
S. Infantis Lairage Swab Slaughter A2 S340
S. Infantis Lairage Swab Slaughter A2 S343
S. Infantis Lairage Swab Slaughter A2 S346
S. Infantis Lairage Swab Slaughter A2 S352
S. Infantis Carcass Swab Slaughter A2 S364
S. Infantis Lairage Swab Slaughter A3 S371
S. Infantis Lairage Swab Slaughter A3 S374
S. Infantis Lairage Swab Slaughter A3 S377
S. Infantis Lairage Swab Slaughter A3 S436
S. Infantis Carcass Swab Slaughter A3 S439
S. Infantis Carcass Swab Slaughter A4 S453
S. Infantis Lairage Swab Slaughter C1 S681
S. Infantis Lairage Swab Slaughter C1 S684
S. Infantis Lairage Swab Slaughter C1 S678
S. Infantis Carcass Swab Slaughter A4 S451
S. Infantis MLN Slaughter A2 S358
S. Infantis Lairage Swab Slaughter C4 S1119
S. Infantis Lairage Swab Slaughter C7 S1310
S. Infantis Carcass Swab Slaughter A3 S386


20
21
22
23
24
25
26
27
28
29
S. Infantis Carcass Swab Slaughter A2 S368
S. Infantis Carcass Swab Slaughter A2 S389
S. Infantis Carcass Swab Slaughter A2 S392
S. Infantis Carcass Swab Slaughter A2 S395
S. Infantis Carcass Swab Slaughter A3 S398
S. Infantis Carcass Swab Slaughter A3 S401
S. Inverness MLN Slaughter A5 S457
S. Inverness MLN Slaughter A5 S515
S. Inverness Truck Swab Nursery1 C3 S542
S. Inverness MLN Slaughter A5 S518
S. Inverness Truck Swab Nursery1 C3 S543
S. Inverness MLN Slaughter A5 S512
S. Braenderup MLN Slaughter A7 S1344
6,7:Nonmotile Lagoon Farrowing C3 S535
S. Typhimurium MLN Slaughter C6 S1176
S. Typhimurium Soil Finishing1 C4 S930
S. Typhimurium Soil Finishing1 C4 S931
S. Anatum Feed Finishing1 A3 S311
S. Newport Water Nursery1 C7 S939
S. Typhimurium Water Nursery1 C7 S938
S. Senftenberg Fecal Nursery1 C4 S617
S. Typhimurium Floor Swab Nursery1 C4 S629
S. Typhimurium MLN Slaughter C4 S1110
S. Typhimurium MLN Slaughter C4 S1111
S. Typhimurium Truck Swab Slaughter C5 S1134
S. Typhimurium Feed Nursery1 C6 S792
S. Typhimurium Feed Finishing2 C10 S1384
S. Typhimurium Floor Swab Finishing2 C10 S1392
S. Typhimurium Fecal Farrowing C4 S545
S. Typhimurium Feed Nursery1 C3 S601
S. Typhimurium Fecal Nursery1 C4 S611
S. Senftenberg MLN Slaughter C10 S1429
S. Senftenberg Fecal Nursery1 C5 S726
S. Senftenberg Feed Nursery1 C6 S797
S. Senftenberg Fecal Farrowing C7 S898
S. Typhimurium Fecal Nursery1 C5 S722
S. Typhimurium Fecal Nursery2 C3 S806
S. Typhimurium Feed Nursery2 C3 S816
S. Typhimurium Lagoon Nursery1 C7 S945
S. Typhimurium Water Finishing2 C10 S1399
S. Typhimurium Feed Nursery1 C4 S623
S. Senftenberg Fecal Nursery1 C4 S616
S. Typhimurium Lagoon Nursery1 C7 S942
S. Typhimurium Lagoon Nursery2 C6 S887
S. Typhimurium Fecal Farrowing C7 S893
S. Typhimurium Lagoon Nursery1 C7 S951
S. Typhimurium Lagoon Nursery1 C7 S953
S. Typhimurium Fecal Nursery2 C3 S800
S. Ouakam MLN Slaughter C10 S1421
S. Ouakam MLN Slaughter C10 S1461


30
31
32
33
34
35
36
37
S. Ouakam Feed Nursery1 C5 S749
S. Ouakam Lagoon Nursery1 C5 S769
S. Ouakam Lagoon Nursery1 C5 S770
S. Ouakam Lagoon Nursery1 C5 S779
S. Ouakam Lagoon Nursery1 C5 S781
S. Ouakam Lagoon Nursery2 C5 S883
S. Ouakam Lagoon Nursery2 C5 S885
S. Ouakam Fecal Nursery1 C6 S786
S. Ouakam Lagoon Nursery1 C7 S943
S. Ouakam Lagoon Nursery1 C5 S780
S. Ouakam Lagoon Finishing1 C7 S1211
S. Ouakam MLN Slaughter C10 S1411
S. Ouakam MLN Slaughter C10 S1425
S. Ouakam Floor Swab Finishing2 C10 S1396
S. Ouakam Lagoon Finishing2 C10 S1405
S. Ouakam Feed Nursery1 C5 S745
S. Ouakam Feed Nursery1 C5 S746
S. Ouakam Fecal Finishing2 C10 S1368
S. Ouakam Fecal Finishing2 C10 S1371
S. Ouakam Fecal Finishing2 C10 S1395
S. Ouakam Floor Swab Finishing2 C10 S1397
S. Ouakam Lagoon Finishing2 C10 S1400
S. Schwarzengrund Feed Finishing1 C6 S967
S. Ouakam Lagoon Nursery2 C5 S878
S. Ouakam Fecal Farrowing C7 S891
S. Ouakam Fecal Farrowing C7 S911
S. Ouakam Floor Swab Farrowing C7 S914
S. Ohio Feed Finishing1 C9 S1228
S. Typhimurium Water Finishing2 C9 S1323
S. Typhimurium var C Feed Finishing2 A1 S270
S. Typhimurium Feed Finishing2 A1 S271
S. Heidelberg Lagoon Nursery1 C3 S607
S. Typhimurium Lagoon Finishing1 C1 S481
S. Anatum Feed Finishing1 C7 S1199
S. Anatum Floor Swab Finishing1 C7 S1202
S. Johannesburg Carcass Swab Slaughter C7 S1298
S. Heidelberg Feed Nursery2 C2 S429
S. Derby Feed Nursery2 C2 S430
S. Infantis Carcass Swab Slaughter C10 S1448
S. Anatum Fecal Farrowing C2 S319
S. Ouakam Floor Swab Finishing1 C6 S970
S. Typhimurium Feed Nursery2 C4 S860
S. Muenchen MLN Slaughter A2 S361
S. Typhimurium Truck Swab Finishing1 C1 S496
S. Give Fecal Nursery1 A5 S258
S. Give Water Nursery1 A5 S263
S. Give Water Nursery1 A5 S266
S. Give Fecal Nursery1 A5 S257
S. Give Water Nursery1 A5 S262
S. Give Water Nursery1 A5 S265



38
39
40
41
42
43
44
45
46
47
48
S. Inverness MLN Slaughter A5 S514
S. Give MLN Slaughter A2 S355
S. Rissen Fecal Nursery2 C3 S812
S. Rissen Feed Nursery2 C3 S823
S. Rissen Floor Swab Nursery2 C3 S824
S. Rissen Water Nursery2 C3 S833
S. Rissen Lagoon Nursery2 C3 S836
S. Rissen Floor Swab Nursery1 C3 S604
S. Rissen Floor Swab Nursery2 C3 S829
S. Rissen Floor Swab Nursery2 C3 S830
S. Rissen Lagoon Nursery2 C3 S841
S. Rissen Floor Swab Finishing2 C3 S1004
S. Rissen Floor Swab Finishing2 C3 S1009
S. Rissen MLN Slaughter C3 S1031
S. Typhimurium Soil Nursery1 C4 S644
S. Infantis Carcass Swab Slaughter C10 S1446
S. Infantis Lairage Swab Slaughter C11 S1449
S. Typhimurium Floor Swab Finishing2 C2 S647
S. Muenchen Fecal Finishing1 C1 S478
S. Muenchen MLN Slaughter A5 S454
S. Muenchen MLN Slaughter A5 S460
S. Muenchen MLN Slaughter A7 S1341
S. Johannesburg Lagoon Nursery1 C5 S782
S. Ohio Fecal Nursery2 C4 S851
S. Ohio Soil Nursery2 C4 S857
S. Ohio Truck Swab Finishing1 C4 S995
S. Ohio Fecal Finishing2 C4 S998
S. Ohio Fecal Finishing2 C9 S1317
S. Ohio Fecal Nursery1 C4 S608
S. Ohio Feed Nursery1 C4 S620
S. Ohio MLN Slaughter C1 S669
S. Ohio Floor Swab Finishing2 C4 S1001
S. Ohio Floor Swab Finishing1 C9 S1232
S. Typhimurium Feed Finishing1 C10 S1326
S. Typhimurium Fecal Farrowing C4 S544
S. Typhimurium Lagoon Finishing1 C6 S977
S. Typhimurium MLN Slaughter C7 S1292
S. Typhimurium Carcass Swab Slaughter C7 S1295
S. Derby Floor Swab Nursery1 C2 S329
S. Derby Floor Swab Nursery1 C2 S324
S. Derby Fecal Nursery2 C2 S427
S. Infantis Lagoon Nursery2 C2 S435
S. Derby Floor Swab Finishing1 C2 S502
S. Derby Lairage Swab Slaughter C7 S1313
S. Derby Floor Swab Nursery2 C2 S432
S. Derby MLN Slaughter C3 S1019
S. Derby Carcass Swab Slaughter C3 S1082
S. Derby Lagoon Finishing2 C3 S1010
S. Derby MLN Slaughter C3 S1048



49
50
51
52
53
54
55
56
57
58
S. Derby Lagoon Finishing2 C3 S1018
S. Derby MLN Slaughter C3 S1034
S. Derby Carcass Swab Slaughter C3 S1085
S. Derby MLN Slaughter C4 S1102
S. Derby Lagoon Finishing2 C3 S1013
S. Derby MLN Slaughter C3 S1023
S. Derby MLN Slaughter C3 S1039
S. Derby MLN Slaughter C3 S1044
S. Derby Carcass Swab Slaughter A1 S302
S. Derby Carcass Swab Slaughter A1 S305
S. Derby Floor Swab Finishing2 C1 S651
S. Derby MLN Slaughter C1 S672
S. Derby Lagoon Finishing2 C1 S657
S. Derby Water Finishing2 C1 S654
S. Infantis Soil Farrowing C2 S322
S. Typhimurium Lagoon Finishing1 C4 S932
S. Cerro Fecal Farrowing C7 S899
S. Cerro Fecal Farrowing C7 S908
S. Cerro Floor Swab Farrowing C7 S916
S. Derby Fecal Farrowing C1 S314
S. Infantis Lagoon Nursery1 C4 S643
S. Mbandaka MLN Slaughter C10 S1438
S. Mbandaka Lagoon Finishing1 C1 S484
S. Mbandaka MLN Slaughter C10 S1436
S. Typhimurium Soil Nursery2 C1 S421
S. Typhimurium Soil Nursery2 C1 S422
S. Typhimurium Lagoon Finishing1 C1 S486
S. Rough_O:r:1,5 Lairage Swab Slaughter A5 S521
S. Mbandaka Truck Swab Slaughter C5 S1138
S. Typhimurium Truck Swab Slaughter C5 S1131
S. Typhimurium Lagoon Nursery1 C7 S948
S. Mbandaka Lagoon Nursery2 C7 S957
S. Typhimurium Lagoon Nursery2 C7 S956
S. Typhimurium Feed Nursery1 C5 S756
S. Typhimurium Lairage Swab Slaughter C9 S1356
S. Typhimurium Lagoon Finishing1 C7 S1214
S. Ohio Floor Swab Finishing1 C9 S1235
S. Typhimurium MLN Slaughter C9 S1350
S. London Truck Swab Nursery1 C3 S539
S. London Lairage Swab Slaughter A5 S524
S. London Lairage Swab Slaughter C6 S1193
S. Newport MLN Slaughter A6 S1335
S. Schwarzengrund Feed Finishing1 C6 S968

3. CHAPTER III: Comparative phenotypic and genotypic characterization of temporally and spatially related non-typhoidal *Salmonella* isolated from human clinical cases, pigs and the environment.

Presented here is the manuscript titled "Comparative phenotypic and genotypic characterization of temporally and spatially related non-typhoidal *Salmonella* isolated from human clinical cases, pigs and the environment", is submitted the present year (2013) and under peer review in the journal of *Foodborne Pathogens and Disease*. Additional data are presented in the Appendix.

3.1 Abstract

Non-typhoidal *Salmonella* (NTS) infections caused by antimicrobial-resistant (AR) strains are of great public health concern. We compared the phenotypic and genotypic relationships among temporally and spatially related AR *Salmonella* isolates (n=1058) representing several predominant serotypes, including *S*. Typhimurium, *S*. Typhimurium var Copenhagen, *S*. Derby, *S*. Heidelberg, *S*. Muenchen, *S*. Schwarzengrund and *S*. Rissen of human clinical (n=572), pig (n=212) and environmental (n=274) origin in North Carolina. Antimicrobial susceptibility testing (AST) was performed using the broth microdilution method and genotypic resistance determinants, including class I and II integrons, were identified. Overall, *Salmonella* isolates exhibited the highest frequency of resistance to tetracycline (TET; 50%), followed by sulfisoxazole (FIS; 36%) and streptomycin (STR; 27%). Amikacin (AMI, 0.7%) and ciprofloxacin (CIP, 0.3%) resistance were only exhibited by human clinical isolates. Multidrug resistance (resistance to ≥ 3 antimicrobials; MDR) was

exhibited highest in isolates from pigs (56%), followed by the environment (32%), and lastly in human clinical isolates (21%). The predominant MDR pattern identified in *S*. Derby was FIS-STR-TET (n=78) while AMP-CHL-FIS-STR-TET was the major pattern detected in serotype *S*. Typhimurium (n=84). We identified 16 different antimicrobial resistance genes, including extended spectrum and AmpC β-lactamases producing genes (bla_{TEM}, bla_{PSE} and bla_{CMY-2}), in all the β-lactam and cephalosporin resistant *Salmonella* isolates from humans, pigs and the environment. We detected class I integrons of 1kb and 1.2kb size (humans, 66%; pigs, 85%; environment, 58%) while Class II integrons of 2kb size were identified only in pig (10%) and environmental (19%) isolates. In conclusion, we detected genotypically diverse *Salmonella* serotypes among humans, pigs and the environment in North Carolina. Detection of a higher frequency of MDR isolates and resistance to ciprofloxacin and AmpC β-lactamases is a concern for clinicians to address *Salmonella* infections.

3.2 Introduction

Antimicrobial resistant (AR) *Salmonella* infections in humans and animals are of great concern in terms of national and global public health. Salmonellosis in humans is still one of the most wide spread foodborne illnesses with 1.4 million illnesses, 15,000 hospitalizations and deaths of more than 500 people each year in the United States (1). Non-typhoidal salmonellosis is generally associated with consumption of contaminated meat and other food products (2, 3). In addition, contact with non-food sources such as contaminated water, direct contact with farm animals and the environment also may result in salmonellosis in humans (4, 5). Non-typhoidal *Salmonella* (NTS) serotypes including *S.* Typhimurium, *S.* Heidelberg, *S*. Infantis, *S*. Muenchen, *S*. Anatum and *S*. Derby are commonly isolated from food animals, retail food products, and other environmental sources, and are responsible for foodborne human salmonellosis in the US (6, 7). In the US, the role of therapeutic and prophylactic antimicrobial use in food animals, development and propagation of AR bacterial populations, and their subsequent transmission to humans through the food chain is under extensive debate (8-10). Emergence and dissemination of drug resistant *Salmonella* strains of human and pig origin, including those that are resistant to cephalosporins and quinolones, have been a major public health concern worldwide (11, 12). In addition, development of multidrug resistance (resistance to ≥ 3 antimicrobials; MDR) among *Salmonella* isolates and their continued maintenance in the absence of selection pressure is especially concerning (10, 13). These MDR isolates often carry resistance genes either on the chromosome or else on mobile genetic elements such as plasmids and integrons (14).

Molecular characterization of AR determinants is important for surveillance and monitoring. Genotyping of *Salmonella* using pulsed-field gel electrophoresis (PFGE) has been used by public health and surveillance agencies for identifying different sources of pathogens, both in outbreak investigations and in epidemiological studies (10, 15, 16).

North Carolina ranks second in pork production in the US, contributing 14.4% of the national inventory (17). In past decade, many *Salmonella* outbreaks in humans associated with pork have been reported worldwide, including the US (7, 18, 19). To the authors' knowledge, no studies have been conducted to compare temporally and spatially related NTS isolates from human clinical cases with *Salmonella* isolates arising from pigs and the environment as part of a longitudinal study. Therefore, the objective of this study was to characterize and compare *Salmonella* isolates from humans, pigs and the environment by antimicrobial susceptibility testing, identification of AR genes and strain genotyping to determine whether temporally and spatially related *Salmonella* isolates from multiple sources in North Carolina were phenotypically and genotypically similar or diverse.

3.3 Materials and methods

3.3.1 *Salmonella* isolate sources

In this present study, a total of 1,058 temporally and spatially related NTS isolates from human clinical cases (n=572), pigs (n=212) and the farm environment (n=274) originating from multiple counties in North Carolina were characterized at the phenotypic and genotypic levels. The human NTS strains were clinical isolates received from the North Carolina State Public Health Laboratory (NCSPHL) during the same period of pig and

environmental sampling. The geographical distribution of NTS isolates represented in Table 3.1. The human clinical isolates (n=572) originated from 72 counties with the majority of isolates coming from Mecklenburg, Wake, Cumberland and Cabarrus counties in North Carolina (n=183). Out of 572 human clinical isolates, 173 (30%) originated from the primary pig producing counties, including Duplin, Johnston, Sampson and Cumberland counties. All the pig (n=212) and the environmental (n=274) isolates originated from the major pig-producing counties mentioned above. The predominant serotype distributions common to the hosts and origin are highlighted in Table 3.1. The *Salmonella* isolates from pigs and their environment were collected as part of longitudinal study conducted from October 2008 to December 2011 on 30 conventional farms at different stages of production from farm to slaughter in North Carolina. The details of the study design, sampling and microbiological methods, estimates of *Salmonella* prevalence in pigs and their environment at farm and slaughter, antimicrobial susceptibility profiles, and their phenotypic and genotypic characterizations has been reported elsewhere (10).

3.3.2 Antimicrobial susceptibility testing (AST)

AST was performed for all *Salmonella* isolates against a panel of 15 antimicrobials via the broth micro-dilution method in a 96-well Sensititre™ plate CMV1AGNF (Trek Diagnostic Systems, Inc., Cleveland, OH). *Salmonella* isolates from pigs and their environment were tested previously by the above mentioned method as part of longitudinal study reported elsewhere (10); human isolates were tested for the first time for the present investigation.

The classes of antimicrobials tested are important in both veterinary and human medicine and include: aminoglycosides (amikacin, AMI; gentamicin, GEN; kanamycin, KAN; streptomycin, STR), β-lactams (ampicillin, AMP; amoxicillin/clavulanic acid, AUG; ceftriaxone, AXO; cefoxitin, FOX; ceftiofur, TIO), quinolones (ciprofloxacin, CIP; nalidixic acid, NAL), folate pathway inhibitors (sulfisoxazole, FIS; trimethoprim/sulfamethaxazole, SXT), phenicols (chloramphenicol, CHL) and tetracyclines (tetracycline, TET). Testing procedure was carried out as described in the previous study (10). The MICs were recorded and breakpoints were determined based on CLSI recommendations (20). The isolates exhibiting resistance to three or more antimicrobials were classified as MDR.

3.3.3 Detection of resistance genes and integrons

All the AR *Salmonella* isolates from humans, pigs and the environment were screened for the presence of all known corresponding resistance genes and class 1 and 2 integrons based on their AR profile. Polymerase chain reaction (PCR) was performed for detection of different AR genes using the following primers, including: the ESBLs bla_{TEM}, $bla_{\mathrm{PSE,}}$ (21) and $bla_{\mathrm{CMY\text{-}2}}$ genes (22), streptomycin resistance coding *aadA1/A2* and *strA/B* genes (23), kanamycin resistance coding *aphAI*, *Kn* genes (24), chloramphenicol resistance coding *cml* gene, sulfisoxazole and trimethoprim/sulfamethoxazole resistance coding *sulI* (25) and *sulII* genes (26), tetracycline resistance coding *tet(A)*, *tet(B)*, *tet(C)*, and *tet(G)* genes, and class1 and class 2 integrons (27). Template DNA was purified using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacture's recommendations. Amplification reactions were carried out as described in the above studies.

3.3.4 Pulsed field gel electrophoresis (PFGE) analysis

A subset of *Salmonella* isolates (pigs, n=46; environment, n=80; humans, n=271) which were purposively chosen to be representative of different sources of origin, serotype, counties and AR profiles were genotyped using PFGE, following the PulseNet protocol (Ribot *et al*., 2006). BioNumerics software version 6.1(Applied Maths, Kortrijk, Belgium) was used to analyze the PFGE images. A dendrogram was generated to determine the clonal relationship among human, pig and environmental isolates using an unweighted-pair group method with average linkages (UPGMA), and with band position tolerance and optimization of 1.5% each.

3.3.5 Statistical analysis

SigmaPlot 11.2 (Systat Software, Inc., Chicago, IL) was used to calculate odds ratios (OR) and 95% confidence intervals (CI) to explore the associations between MDR and the predominant serotypes. A *P*-value of 0.05 or lower was considered statistically significant.

3.4 Results

3.4.1 Antimicrobial resistance profiles

A total of 1,058 *Salmonella* isolates from humans (n=572), pigs (n=212) and their environment (n=274) were tested against a panel of 15 antimicrobials. The overall MIC levels and frequency of AR of *Salmonella* isolates are presented in Table 3.2 and Figure 3.1. All the *Salmonella* isolates, irrespective of source of origin, exhibited similar MIC_{50} and MIC_{90} with the exceptions being: TET MIC_{50} in humans which was 4 µg/mL (versus pigs and environment: 32 µg/mL); FIS MIC_{50} in pigs which was 256 µg/mL (humans and

environment, 64 µg/mL) and CHL MIC_{90} in environmental isolates which was 16 µg/mL (pigs and humans, 32 µg/mL). Overall, *Salmonella* isolates exhibited the highest frequency of resistance to tetracycline (TET; 50%), followed by sulfisoxazole (FIS; 36%) and streptomycin (STR; 27%). In addition, *Salmonella* isolates from human clinical cases exhibited slightly higher frequencies of resistance to cephalosporins including, AXO, FOX and TIO (3% each) compared to pigs and environmental isolates (2% each). Overall, the frequency of AR was higher in *Salmonella* isolates of pigs origin (82.6%) followed by environmental (67.6%) and human clinical isolates (28%), with the exception of AMI (0.7%) and CIP (0.3%) resistance which were only exhibited by human clinical isolates.

3.4.2 MDR patterns identified

The major MDR patterns identified in humans, pigs and their environment are represented in Table 3. The highest frequency of MDR patterns were detected in *Salmonella* isolated from pigs (55.6%) compared to the environment (32.4%) and human (21.3%). The most common MDR patterns identified in all the three sources were FIS-STR-TET, and AMP-CHL-FIS-STR-TET associated with *S*. Derby and *S*. Typhimurium respectively. The *S*. Derby serotype was significantly ($P<0.001$) associated with an MDR pattern FIS-STR-TETwith an OR of 27 (95% CI=15.6-46.8) compared to *S*. Typhimurium isolates with this MDR pattern. Specific MDR patterns were detected in particular hosts including FIS-STR-TET, FIS-SXT-TET and AMP-AUG-AXO-FOX-TIO-TET in *S*. Typhimurium isolated from pigs and environmental isolates. In contrast, the AMP-KAN-STR-TET (n=5) pattern was found only in *S*. Heidelberg isolates of human origin (Table 3.3). A single human clinical *S*. Typhimurium isolate was resistant to 12 antimicrobials out of 15 tested with an MDR pattern

of AMI-AXO-CHL-CIP-FOX-FIS-GEN-KAN-NAL-STR-TIO-TET; very importantly, including resistance against both quinolones and multiple cephalosporins.

3.4.3 Molecular characterization of antimicrobial resistance (AR) determinants

Using PCR, we identified 16 different AR genes conferring resistance to a number of classes of antimicrobials. Ampicillin resistance was predominantly encoded by the extended spectrum β- lactamase (ESBL) producing bla_{TEM} gene (humans, 71%; pigs, 49%; environment, 81%), followed by the bla_{PSE} gene (humans, 17%; pigs, 49%; environment,11%). All the *Salmonella* isolates from humans, pigs and the environment that were resistant to 3rd generation cephalosporins carried the AmpC β-lactamases producing bla_{CMY-2} gene. Tetracycline resistant isolates from pigs and the environment harbored four different genes, predominantly *tet(A)* (pigs, 72%; environment, 68%) followed by *tet(B)* (pigs, 10%; environment, 19%), *tet(C)* (pigs, 4%; environment, 5%) and *tet(G)* (pigs, 15%; environment, 9%) genes. Among *Salmonella* isolates from humans, tetracycline resistance was predominantly encoded by *tet(G)* (58%), *tet(A)* and *(C)* (each at 17%), and with the notable absence of the *tet(B)* gene. Common MDR patterns in humans, pigs and the environment and associated AR genes are represented in Table 3.3. The *S.* Typhimurium penta-resistant (AMP-CHL-FIS-STR-TET) isolates from humans, pigs and the environment harbored similar resistant genes, including bla_{TEM} / bla_{PSE}, *cml*A, *aadA1/A2* and *tet(G)*, encoding resistance to AMP-CHL-STR-TET, respectively (Table 3.3). The FIS encoding gene *sul2* was only detected in the isolates exhibiting these two resistance patterns (FIS-STR-TET and FIS-SXT-TET) (Table 3). We detected class I integrons of 1kb and 1&1.2kb size (humans, 66%; pigs, 85%; environment, 58%) and class II integrons of 2kb (humans, 0%;

pigs, 10%; environment, 19%) in MDR *Salmonella* isolates from pigs and the environment. All the penta-resistant *S*. Typhimurium from each sources exhibited class I integrons of 1&1.2kb size.

3.4.4 Pulsed Field Gel Electrophoresis (PFGE)

Genotyping of (n=397) temporally and spatially related *Salmonella* isolates by PFGE with the *Xba*I restriction enzyme distributed them into 74 major clusters consisting of isolates with similar fingerprint profiles, and another 118 unique PFGE patterns represented by a single isolate each (data not shown). *S*. Muenchen isolates from each source had more diversified fingerprint profiles compare to other study serotypes. All the *S*. Heidelberg isolates were closely related based on fingerprint profile in a dendrogram and distributed into one big cluster with 80% genotypic similarity.

Two separate dendrograms were created for *S*. Derby and *S*. Typhimurium representing similar counties and AR patterns. Identical fingerprint profiles were found among *S*. Derby isolates of pig and environmental origin from three major pig producing counties including Cumberland, Johnston and Sampson (cluster 3-5; Figure 3.2). All the *S*. Derby isolates with MDR pattern FIS-STR-TET from the three sources were clustered closely in the dendrogram (cluster 2-6; Figure 3.2). Even though the *S*. Derby from humans (ID HS749 and HS289) had identical resistance pattern (FIS-STR-TET) as those that originated from pigs and the environment in the same county (Sampson and Cumberland), they had different fingerprint profiles (Figure 3.2). Phenotypic similarity was not always replicated at the genotypic level. The majority of the *S*. Typhimurium isolates had an identical fingerprint profile within and between the sources of origin. Genotypic (100%) and

phenotypic similarity based on MDR pattern (AMP-CHL-FIS-STR-TET) among isolates of human, pig and environmental origin was detected only among the *S*. Typhimurium serotype (cluster 1 and 2; Figure 3.3). It is worthwhile to mention that the human *S*. Typhimurium isolates in these clusters were reported from counties (Cumberland and Wake) adjacent to the major pig producing counties of North Carolina. Overall, the isolates from human, pigs and the environment exhibited higher levels of genotypic diversity among *S*. Derby, *S*. Heidelberg and *S*. Muenchen isolates and much more genotypic similarity in *S*. Typhimurium isolates.

3.5 Discussion

The objective of this study was to compare temporally and spatially related non-typhoidal *Salmonella* isolates from humans, pigs and the environment based on their AR phenotypes and genotypic fingerprint profiles. The frequency of AR was higher in *Salmonella* isolates from pigs (82.6%) and their environment (67.6%) compared to human clinical *Salmonella* isolates (28%). In addition, we observed a higher MIC_{50} (32µg/mL) for TET in pig and environmental isolates compare to the MIC_{50} (4µg/mL) for human isolates. One reason could be that the pig and environmental isolates were from conventional production systems which routinely use antimicrobials for prophylaxis (tetracyclines as growth promoter) and therapeutic purposes, thereby contributing to an increased frequency of resistance and MIC to antimicrobials (10).

Resistance to β-lactams, including ampicillin and cephalosporins, was observed at low levels in human, pig and environmental isolates. In addition, human clinical *Salmonella*

isolates exhibited resistance to CIP (0.3%), which was never observed in pig or environmental isolates. Resistance to CIP in *Salmonella* isolates of humans appears to be increasing every year based on the NARMS annual report on AR of NTS isolates from 1999-2010, as well as other studies (16, 28-30). Resistance among *Salmonella* to ciprofloxacin and 3rd generation cephalosporins is concerning since they are the drugs of choice for treating human invasive *Salmonella* infections. We observed a higher MIC_{50} (32µg/mL) for TET in pig and environmental isolates compare to the MIC_{50} (4µg/mL) for human isolates. One reason could be the extensive use of tetracylines in feed as growth promoter and prophylaxis, and also for therapeutics in the swine farms that were sampled as part of a longitudinal study (10).

A higher frequency of MDR isolates was observed in *Salmonella* isolates of pigs (55.6%) and their environment (32.4%) compared to humans (21.3%). According to the NARMS executive report (1999-2010), the number of reported NTS MDR isolates in humans dropped to 9% from 2007-2010. Interestingly, in our study the frequency of MDR isolates from human clinical NTS isolates was higher (21.3%) compared to recent NARMS data (29). This variation could be attributed to regional differences. The two most common patterns observed in this study among all the three sources were FIS-STR-TET and AMP-CHL-FIS-STR-TET. The *S*. Derby serotype was 27 times (odds ratio) more likely to be associated with the FIS-STR-TET pattern than *S*. Typhimurium which suggests establishment of this serotype with a specific MDR pattern in this region. Emergence of *S*. Derby with this specific MDR pattern is concerning as it is one of the top ten serotypes isolated both from pigs and humans (29). Importantly, none of the three classes of

antimicrobial to which it is resistant are currently considered critically important by the WHO (31). The MDR pattern AMP-CHL-FIS-STR-TET was detected at a higher frequency among the human *S*. Typhimurium isolates when compared to pigs and their environment. This penta-resistant pattern is specific to *S*. Typhimurium phage type DT104 and commonly associated with clinical salmonellosis and human foodborne outbreaks worldwide (11, 32, 33). This penta-resistant phenotype is often a component of higher-order MDR patterns that include resistance elements against the critically important fluoroquinolones and 3rd and 4th generation cephalosporins. As an example, a single *S*. Typhimurium isolate from a clinical human case exhibited resistance to 12 antimicrobials, including three different cephalosporins and ciprofloxacin. This pattern in *S.* Typhimurium has been previously reported from human clinical cases (3, 28). Infections with this strain are concerning as they can lead to treatment failure and result in more severe clinical outcomes.

The molecular characterization of these isolates using PCR to detect the presence of genes encoding resistance to various antimicrobials was consistent among the *Salmonella* isolates from all three sources. Detection of the AmpC β-lactamases producing gene bla_{CMY-2} among all the *Salmonella* isolates resistant to 3rd generation cephalosporins, including AXO and TIO, was in agreement with previous reports (22, 34). This is the first report of AmpC β-lactamases encoding bla_{CMY-2} genes in *Salmonella* from this region and that AmpC β-lactamases resistant *Salmonella* isolates are predominantly encoded by bla_{CMY-2} in NC. Presence of this gene is concerning because *Salmonella* isolates carrying these genes are at an increased risk of acquiring resistance to other classes of antimicrobials (3, 35).

The majority of penta-resistance in *S*. Typhimurium was encoded by resistance genes carried on class I integrons and these are believed to play an important role in the dissemination of AR among susceptible populations of *Salmonella* both in humans and animals (14). This was evidenced by detection of class I integrons of 1& 1.2kb in all the penta-resistant (AMP-CHL-FIS-STR-TET) *S*. Typhimurium isolates of human, pig and the environment. All these isolates carried similar resistance genes, including: bla_{TEM} / bla_{PSE}, *cml*A, *aadA1/A2* and *tet(G)*. All the *S*. Derby with FIS-STR-TET pattern carried a class I integron of 1kb size suggesting too that this serotype could play a potentially important role in transmission of AR to susceptible population as for *S*. Typhimurium. We identified the rarely reported class II integrons of 2kb size in pig and environmental *Salmonella* isolates. To the best of our knowledge, this is the first report of class II integrons in *Salmonella* in this region. The class II integrons will have additional gene cassettes which encode resistance to trimethoprim/sulfamethaxazole. Further analysis of the class II integrons identified in our study will be performed later to identify other resistant gene cassettes that may be present in them.

A subset of *Salmonella* isolates from humans, pigs and the environment exhibiting similar AR patterns and originating from pig producing and non-pig producing counties were genotyped using PFGE. We identified 74 major clusters consisting of isolates with similar fingerprint profiles and an additional 118 unique profiles. Isolates of *S*. Muenchen from human clinical cases that originated from the same county were grouped into two different clusters. However, the majority of *S*. Muenchen isolates from humans had a unique fingerprint profile, which has previously been reported in *S*. Muenchen and other *Salmonella*

serotypes (28, 36). This diversity suggests that these serotypes could potentially originate from multiple sources in this region. Similarly, *S*. Derby isolates from humans were more diverse compared to pig and environmental isolates even though they had similar resistance patterns and were isolated from counties adjacent to major pig producing counties; this suggests that different genotypes of *S*. Derby are prevalent in NC. We identified 100% genotypic similarity only in penta-resistant *S*. Typhimurium isolates from humans, pigs and the environment originating from the major pig producing counties of North Carolina (cluster 1 and 2; Figure 3.3). However, it is important to mention that no human *Salmonella* outbreak associated with pork has been reported from this area.

3.6 Conclusion

In conclusion, this is the first report to compare temporally and spatially related non-typhoidal *Salmonella* isolated from humans, pigs and the environment from this region of the US. Increased frequency of resistance to cephalosporins and ciprofloxacin in human clinical isolates will further challenge clinicians attempting to treat invasive *Salmonella* infections. Detection of similar MDR patterns, Class I and II integrons in *Salmonella* of pigs, their environment and of human origin is a growing public health concern. Even though the human isolates were not directly linked to pigs/farm environment, identical fingerprint profiles suggest that the same strains are circulating in NC. Further studies to determine the role played by different reservoirs in determining the occurrence and dissemination of AR *Salmonella* in the food chain will be key to identifying these sources.

3.7 Acknowledgments

We thank the swine producers and swine processing facilities in NC for allowing access to their facilities and the NCSLPH for providing human clinical *Salmonella* strains. This work was funded by the USDA National Research Initiative grant (2008-529245), USDA National Integrated Food Safety Initiative grant (2008-529461) and National Pork Board grant (555105).

3.8 Disclosure Statement

No competing financial interest exists.

3.9 References

1. Scallan E, Hoekstra R, Angulo F, Tauxe R, Widdowson M, Roy S, Jones J, Griffin P. 2011. Foodborne illness acquired in the United States - Major pathogens. Emerg. Infect. Dis. 17:7-15.
2. Foley SL, Lynne AM. 2008. Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. J. Anim. Sci. 86:E173-187.
3. Glenn L, Lindsey R, Folster J, Pecic G, Boerlin P, Gilmour M, Harbottle H, Zhao S, McDermott P, Fedorka-Cray P, Frye J. 2013. Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Canada. Microb. Drug. Res.19:175-184.
4. Hendriksen S, Orsel K, Wagenaar J, Miko A, van Duijkeren E. 2004. Animal-to-human transmission of *Salmonella* Typhimurium DT104A variant. Emerg. Infect. Dis. 10:2225-2227.
5. Hoelzer K, Switt A, Wiedmann M. 2011. Animal contact as a source of human non-typhoidal salmonellosis. Vet. Res. 42.
6. Foley SL, Lynne AM, Nayak R. 2008. *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. J. Anim. Sci. 86:E149-162.
7. CDC. 2007. The food borne outbreak online database (Food). Available online at http://wwwn.cdc.gov/foodborneoutbreaks/Default.aspx. Center for Disease Control and Prevention.
8. Alali WQ, Scott HM, Christian KL, Fajt VR, Harvey RB, Lawhorn DB. 2009. Relationship between level of antibiotic use and resistance among *Escherichia coli*

isolates from integrated multi-site cohorts of humans and swine. Prev. Vet. Med. 90:160-167.

9. Quintana-Hayashi MP, Thakur S. 2012. Longitudinal study of the persistence of antimicrobial-resistant *Campylobacter* strains in distinct swine production systems on farms, at slaughter, and in the environment. Appl. Environ. Microbiol. 78:2698-2705.

10. Keelara S, Scott HM, Morrow WM, Gebreyes WA, Correa M, Nayak R, Stefanova R, Thakur S. 2013. Longitudinal study comparing the distribution of phenotypically and genotypically similar antimicrobial resistant *Salmonella* serovars between pigs and their environment in two distinct swine production systems. Appl. Environ. Microbiol.79: doi: 10.1128/AEM.01419-13**.**

11. Gebreyes W, Thakur S, Dorr P, Tadesse D, Post K, Wolf L. 2009. Occurrence of spvA virulence gene and clinical significance for multidrug-resistant *Salmonella* strains. J. Clin. Microbiol. 47:777-780.

12. Vo A, van Duijkeren E, Gaastra W, Fluit A. 2010. Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolates from Vietnam. Plos One 5:e9440.

13. Gebreyes W, Thakur S, Morrow W. 2006. Comparison of prevalence, antimicrobial resistance, and occurrence of multidrug-resistant *Salmonella* in antimicrobial-free and conventional pig production. J. Food. Prot. 69:743-748.

14. Aarestrup FM, Oliver Duran C, Burch DG. 2008. Antimicrobial resistance in swine production. Anim. Health. Res. Rev. 9:135-148.

15. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne. Pathog. Dis. 3:59-67.

16. Dorr P, Tadesse D, Zewde B, Fry P, Thakur S, Gebreyes W. 2009. Longitudinal study of *Salmonella* dispersion and the role of environmental contamination in commercial swine production systems. Appl. Environ.Microbiol. 75:1478-1486.

17. USDA. September 2009. Organic agriculture: organic market overview. U.S. Department of Agriculture, Economic Research Service, Washington, DC. http://www.ers.usda.gov/briefing/organic/demand.htm.

18. Wojcik O, Kjelso C, Kuhn K, Muller L, Jensen T, Kjeldsen M, Ethelberg S. 2012. *Salmonella* Typhimurium outbreak associated with smoked pork tenderloin in Denmark, January to March 2011. Scand. J. Infect. Dis. 44:903-908.

19. Scavia G, Ciaravino G, Luzzi I, Lenglet A, Ricci A, Barco L, Pavan A, Zaffanella F, Dionisi A. 2013. A multistate epidemic outbreak of *Salmonella* Goldcoast infection in humans, June 2009 to March 2010: the investigation in Italy. Euro. Surveill. 18:23-31.

20. CLSI. 2010. Performance Standards for Antimicrobial Susceptibility Testing;Twentieth Informational Supplement, p. 40-45, M100-S20. CLSI, Wayne, PA.

21. Carlson S, Bolton L, Briggs C, Hurd H, Sharma V, Fedorka-Cray P, Jones B. 1999. Detection of multiresistant *Salmonella* Typhimurium DT104 using multiplex and fluorogenic PCR. Mol. Cell. Probes. 13:213-222.

22. Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD. 2001. Identification and expression of cephamycinase bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents. Chemother. 45:3647-3650.

23. Madsen L, Aarestrup F, Olsen J. 2000. Characterisation of streptomycin resistance determinants in Danish isolates of *Salmonella* Typhimurium. Vet. Microbiol. 75:73-82.

24. Frana T, Carlson S, Griffith R. 2001. Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in *Salmonella enterica* serotype Typhimurium phage type DT104. Appl. Environ. Microbiol. 67:445-448.

25. Briggs C, Fratamico P. 1999. Molecular characterization of an antibiotic resistance gene cluster of *Salmonella* Typhimurium DT104. Antimicrob.Agents Chemother.43:846-849.

26. Aarestrup FM, Lertworapreecha M, Evans MC, Bangtrakulnonth A, Chalermchaikit T, Hendriksen RS, Wegener HC. 2003. Antimicrobial susceptibility and occurrence of resistance genes among *Salmonella enterica* serovar Weltevreden from different countries. J. Antimicrob. Chemother. 52:715-718.

27. Ng L, Mulvey M, Martin I, Peters G, Johnson W. 1999. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob. Agents. Chemother. 43:3018-3021.

28. Patchanee P, Zewde B, Taclesse D, Hoet A, Gebreyes W. 2008. Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg isolated from humans and animals. Foodborne Pathog. Dis. 5:839-851.

29. NARMS. 2010. National Antimicrobial Resistance Monitoring System:executive report 2010, Atlanta, Georgia, U.S. Department of Health and Human Services, CDC.

30. Van Boxstael S, Dierick K, Van Huffel X, Uyttendaele M, Berkvens D, Herman L, Bertrand S, Wildemauwe C, Catry B, Butaye P, Imberechts H. 2012. Comparison of antimicrobial resistance patterns and phage types of *Salmonella* Typhimurium isolated from pigs, pork and humans in Belgium between 2001 and 2006. Food. Res. Int. 45:913-918.

31. WHO. 2012. World Health Organization. Critically important antimicrobials for humans medicine, Geneva, Switzerland.

32. Helms M, Ethelberg S, Molbak K, Grp DS. 2005. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emerg. Infect. Dis. 11:859-867.

33. Medalla F, Hoekstra R, Whichard J, Barzilay E, Chiller T, Joyce K, Rickert R, Krueger A, Stuart A, Griffin P. 2013. Increase in resistance to ceftriaxone and nonsusceptibility to ciprofloxacin and decrease in multidrug resistance among *Salmonella* strains, United States, 1996-2009. Foodborne Pathog. Dis. 10:302-309.

34. Van TT, Nguyen HN, Smooker PM, Coloe PJ. 2012. The antibiotic resistance characteristics of non-typhoidal *Salmonella enterica* isolated from food-producing animals, retail meat and humans in South East Asia. Int. J. Food. Microbiol. 154:98-106.

35. Hamilton R, Hulsebus H, Akbar S, Gray J. 2012. Increased resistance to multiple antimicrobials and altered resistance gene expression in CMY-2-positive *Salmonella enterica* following a simulated patient treatment with ceftriaxone. Appl. Environ. Microbiol. 78:8062-8066.

36. Gebreyes W, Thakur S. 2005. Multidrug-resistant *Salmonella enterica* serovar Muenchen from pigs and humans and potential interserovar transfer of antimicrobial resistance. Antimicrob. Agents. Chemother. 49:503-511.

Table 3.1 Geographical distribution of *Salmonella* isolates from humans, pigs and the environment.

Serotypes[a]	**Host and county distribution**[b]		
	Humans (n=572)	**Pigs (n=212)**	**Environment (n=274)**
***S*. Typhimurium** (H=290; P=126; E=184)	Alamance (5), Anson (4), Brunswick (3), Cabarrus (9), Chatham (3), Columbus (3), Carven (8), Cumberland (17), Durham (14), Edgecombe (3), Forsyth (7), Franklin (5), Gaston (5), Guilford (11), Harnett (4), Johnston (6) Mecklenburg (46), Moore (5), Nash (4), Onslow (8), Orange (3), Out of State (6), Pender (3), Randolph (4), Robeson (5), Rowan (3), Stanly (9), Unknown (8), Wake (29), Wilson (4)	Bladen (7), Cumberland (6), Duplin (3), Johnston (20), Sampson (90)	Bladen (3), Cumberland (8), Duplin (10), Johnston (45), Sampson (116)
***S*. Typimurium Var C** (H=135; P=2; E=0)	Alamance (5), Cabarrus (6), Chatham (3), Columbus (3), Carven (7), Cumberland (4), Durham (3), Johnston (3), Mecklenburg (11), Onslow (4), Randolph (5), Richmond (3), , Rockingham (5), Robeson (3), Union (4), Unknown (7), Wake (17), Wilson (4)	N/A	N/A
***S*. Derby** (H=12; P=74; E=32)	Pender (3)	Johnston (4), Sampson (70)	Johnston (26), Sampson (6)
***S*. Heidelberg** (H=50; P=0; E=17)	Guilford (4), Mecklenburg (7), Out of State (3), Vance (3), Wake (3)	N/A	Johnston (16)
***S*. Muenchen** (H=79; P=3; E=0)	Cabarrus (9), Forsyth (3), Mecklenburg (13), New Hanover (8), Onslow (3), Pender (4), Robeson (3), Rutherford (3), Wake (5)	Johnston (3)	N/A
***S*. Rissen** (H=1; P=7; E=39)	N/A	Cumberland (6),	Cumberland (33), Sampson (6)

Table 3.1 (continued)

[a]*S.*Schwarzengrund isolates (H=5; P=0; N=2) county data is not represented since none of the county had ≥ 3 isolates each

H: Human, P: Pig, E: Environment.

[b]Only the counties represents ≥ 3 isolates are listed in the table

Table 3.2 Minimum inhibitory concentration (MIC) levels and frequency of resistance of *Salmonella* isolates from humans, pigs and environment.

Source	Range[a]	AMP	AUG	AXO	FOX	TIO	CHL	CIP	FIS	KAN	NAL	STR	SXT	TET
Humans (n=572)	MIC 50	1	1/0.5	0.25	2	1	8	0.02	64	8	4	32	0.12/2.38	4
	MIC 90	32	16/8	0.25	4	1	32	0.02	256	8	4	64	0.12/2.38	32
	% R	**18**	**2.6**	**3.4**	**3.3**	**3.1**	**12.4**	**0.3**	**19**	**5.9**	**1.3**	**19.5**	**0.8**	**20**
Pigs (n=212)	MIC 50	1	1/0.5	0.25	4	1	8	0.02	256	8	4	32	0.12/2.38	32
	MIC 90	32	16/8	0.25	4	1	32	0.02	256	8	4	64	0.12/2.38	32
	% R	**17.4**	**1.4**	**1.8**	**2.3**	**1.8**	**13.2**	**0**	**68.0**	**1.4**	**4.2**	**48.5**	**4.7**	**82.0**
Environment (n-274)	MIC 50	1	1/0.5	0.25	4	1	8	0.02	64	8	4	32	0.12/2.38	32
	MIC 90	32	16/8	0.25	4	1	16	0.03	256	8	4	64	0.12/2.38	32
	% R	**19.3**	**2.5**	**1.6**	**2.1**	**1.6**	**8**	**0**	**46**	**5.8**	**3.6**	**27.3**	**6.2**	**88.0**

[a]MIC: minimum inhibitory concentration, % R: percent resistance

[b]Antimicrobials: AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

Table 3.3 Distribution of predominant MDR patterns and AR genes in humans, pigs and environment.

Serotype	MDR Patterns[a]	Humans (%), AR genes[b]	Pigs (%), AR genes	Environment (%), AR genes
S. Typhimurium (H=132, P=59, E=60)*	FIS-STR-TET	0 N/A	9 (15) *aad A1/A2, strA/B, sul*2, *tet* (A), class I integrons	7 (12) *aad A1/A2*, *sul*2, *tet* (A), class I integrons
	FIS-SXT-TET	0 N/A	9 (15) *sul*2, *tet* (A), class II *Int*	16 (26) *tet* (A), class II *Int*
	AMP-AUG-AXO-FOX-TIO-TET	0 N/A	3 (5) bla_{TEM}, bla_{CMY-2}, *tet* (C)	5 (8) bla_{TEM}, bla_{CMY-2}, *tet* (C)
	AMP-CHL-FIS-STR-TET	43 (32)	21 (35)	20 (33)
S. Heidelberg (H =29, P=0, E=12)	KAN-STR-TET	9 (31) *kn*, *str A/B*, *tet* (C)	0 N/A	11 (91) *kn*, *str A/B*, *tet* (B)
	AMP-KAN-STR-TET	5 (17) bla_{TEM}, *str A/B*, *tet* (C)	0 N/A	0 N/A
S. Derby (H=6, P=57, E=17)	FIS-STR-TET	6 (100) *aad A1/A2*, *sul*2, *tet* (A), class I *Int*	56 (98) *aad A1/A2*, *sul*2, *tet* (A), class I	16 (98) *aad A1/A2*, *sul*2, *tet* (A), class I *Int*

Table 3.3 (continued)

*H: MDR *Salmonella* isolates representing humans, P: MDR *Salmonella* isolates representing pigs

E: MDR *Salmonella* isolates representing environment

[a]Antimicrobials: AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

[b]bla_{TEM} / bla_{PSE} : genes encoding ampicillin, *cmlA*: chloramphenicol, *aadA1/A2* and *str A/B*: streptomycin, *tet* (A) (B) (C): tetracycline, *kn*: kanamycin, $bla_{CMY\text{-}2}$: extended spectrum β-lactamases.

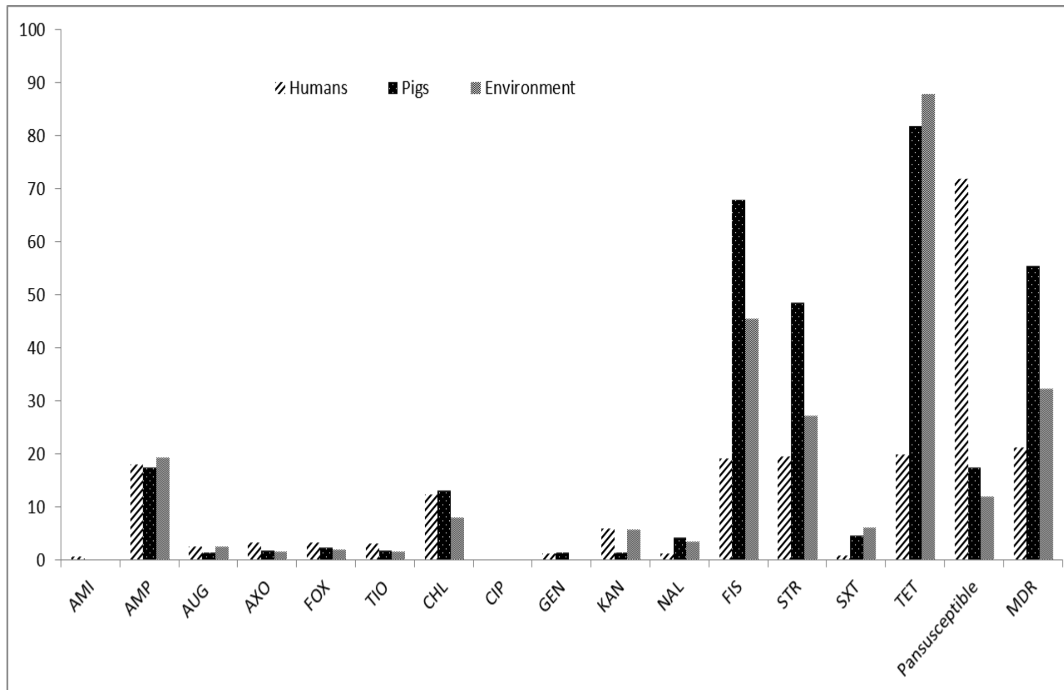


Figure 3.1 Antimicrobial resistance profile of *Salmonella* isolates from humans, pigs and environment.

[a]Antimicrobials: AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

MDR: Multi-drug resistance (resistance ≥ 3 antimicrobials)

Panssuscptible: susceptible to all the antimicrobials tested

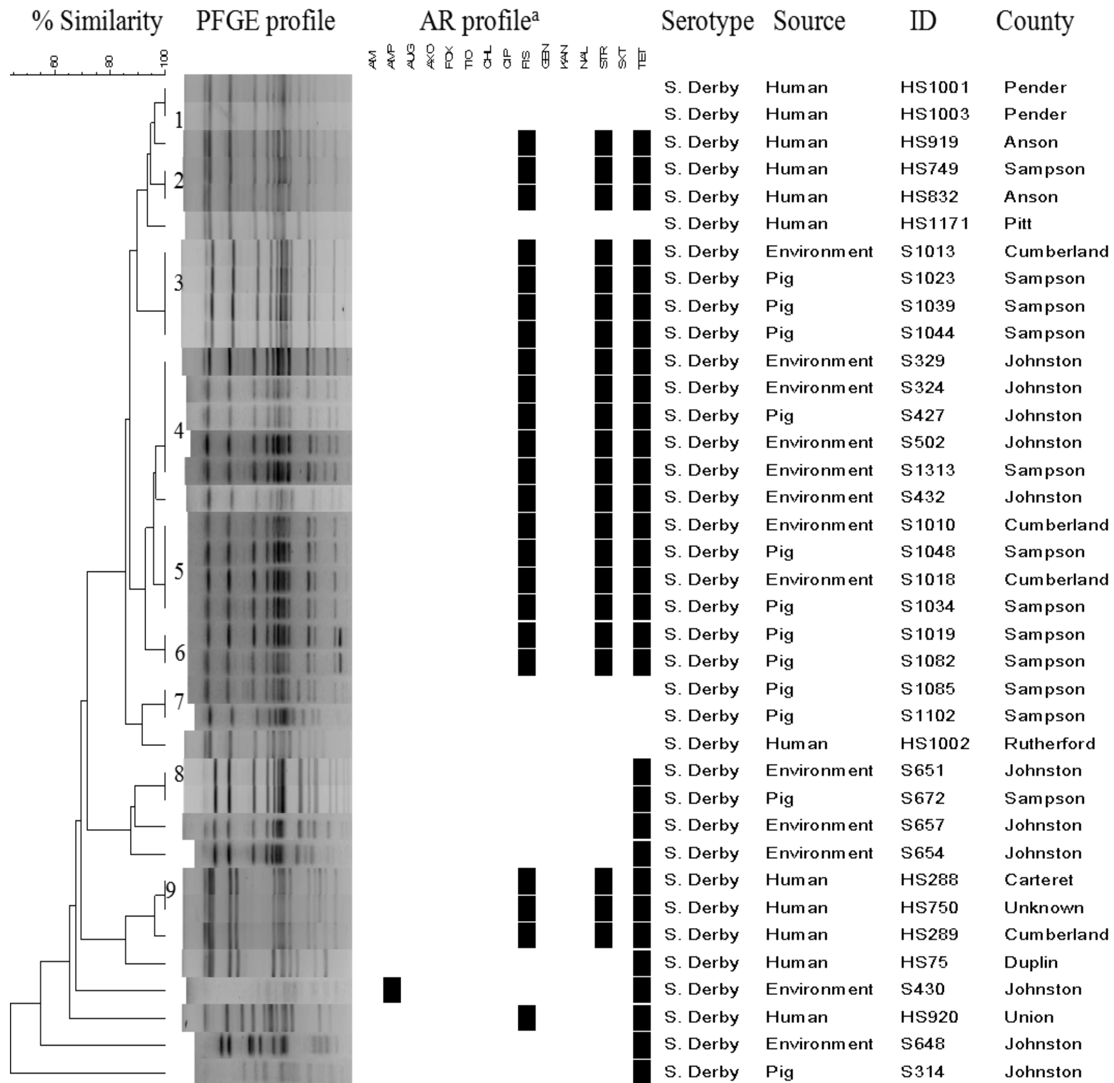


Figure 3.2 Dendrogram showing genotypic similarity among *Salmonella* Derby isolated from humans, pigs and environment.

[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

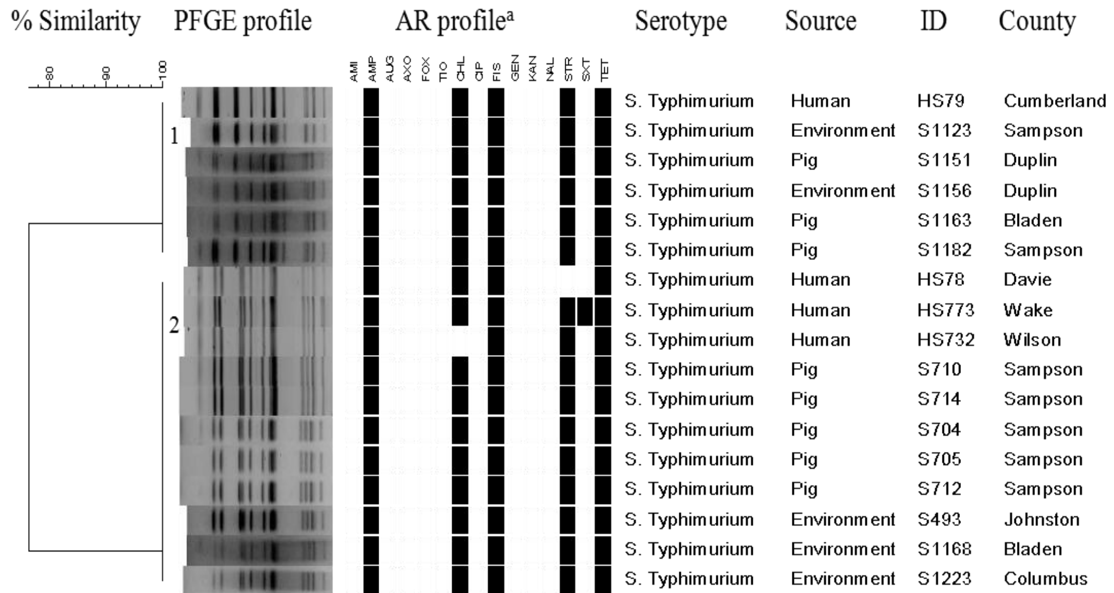
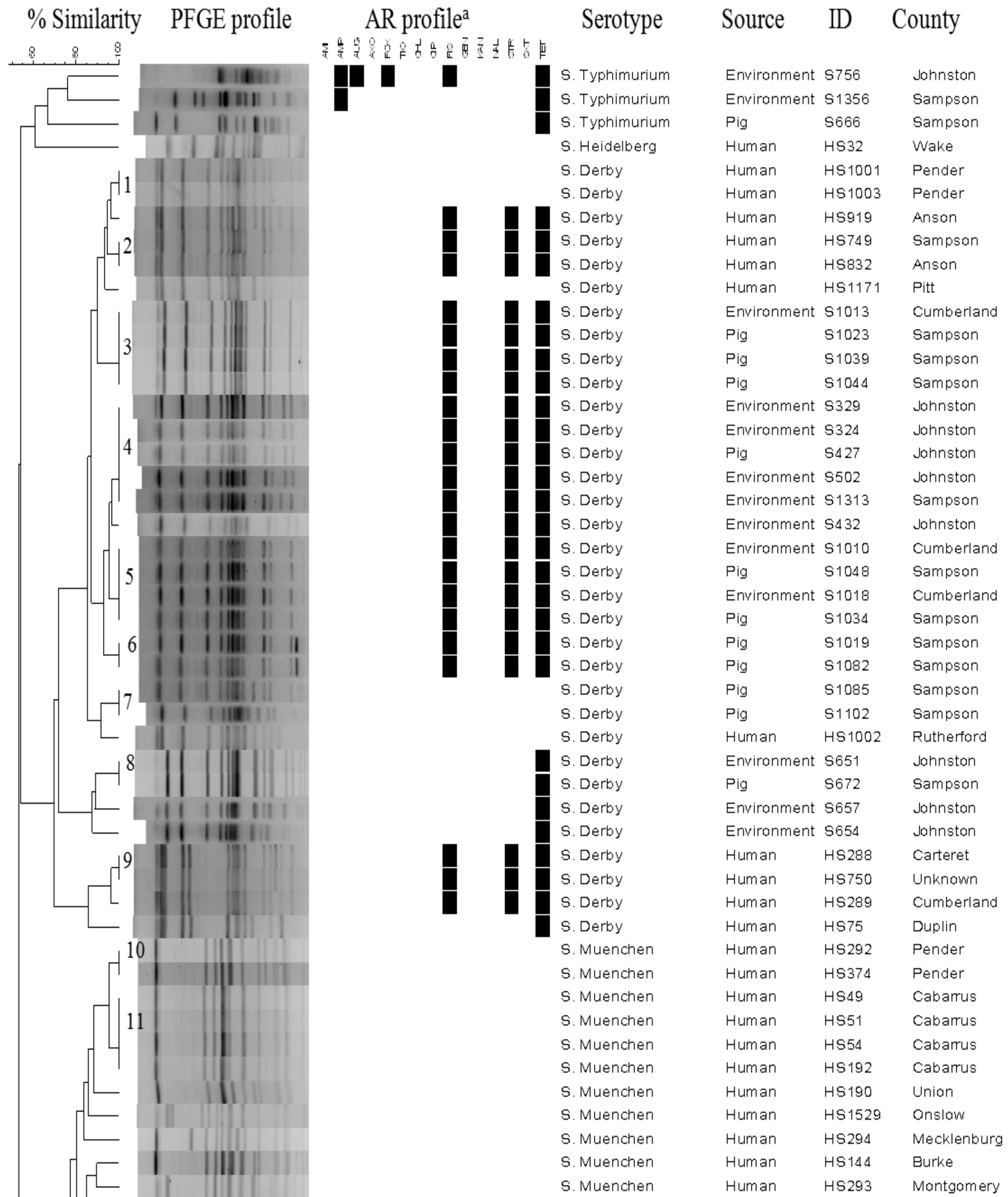


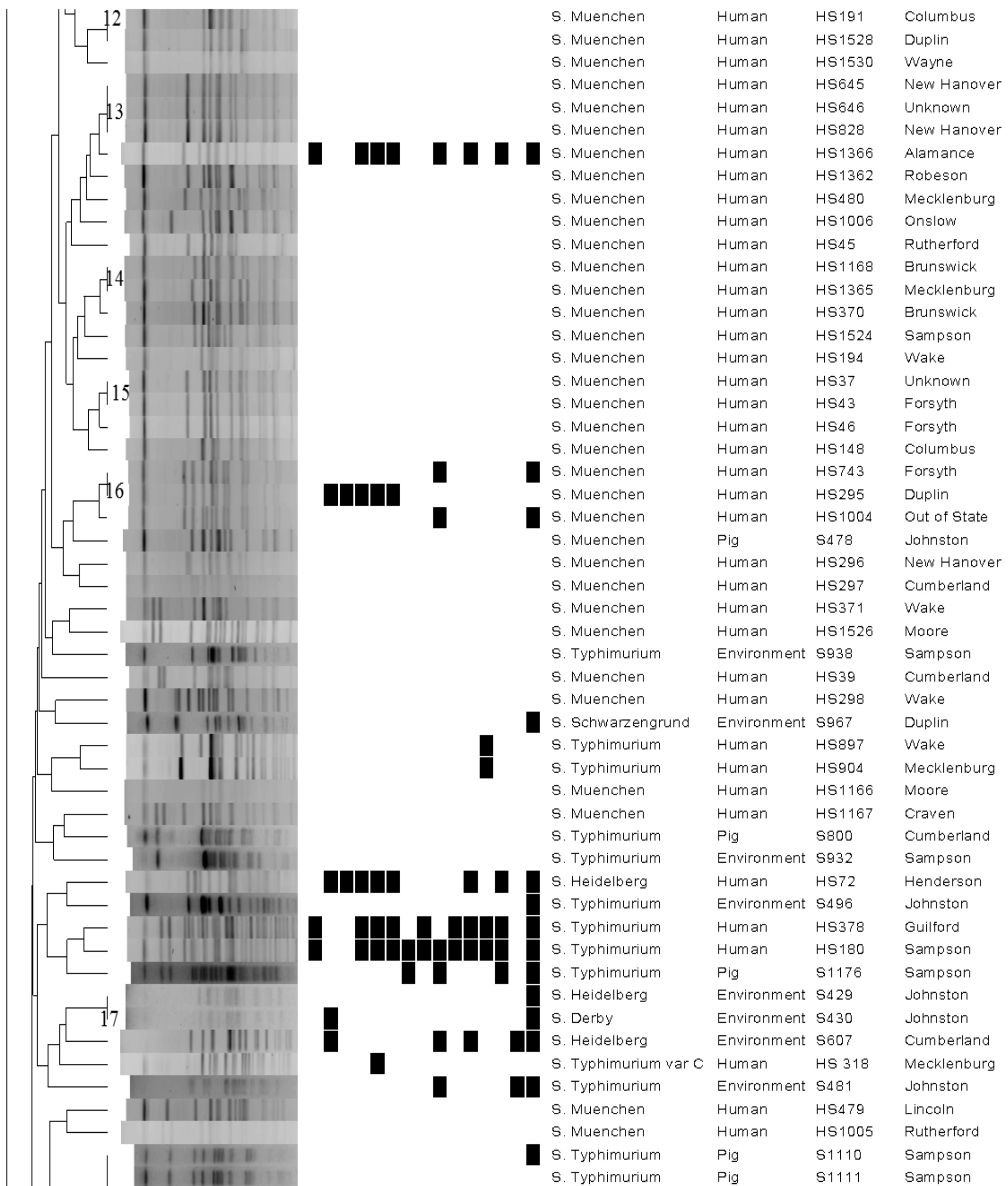
Figure 3.3 Dendrogram showing genotypic similarity among *Salmonella* Typhimurium isolated from humans, pigs and environment.

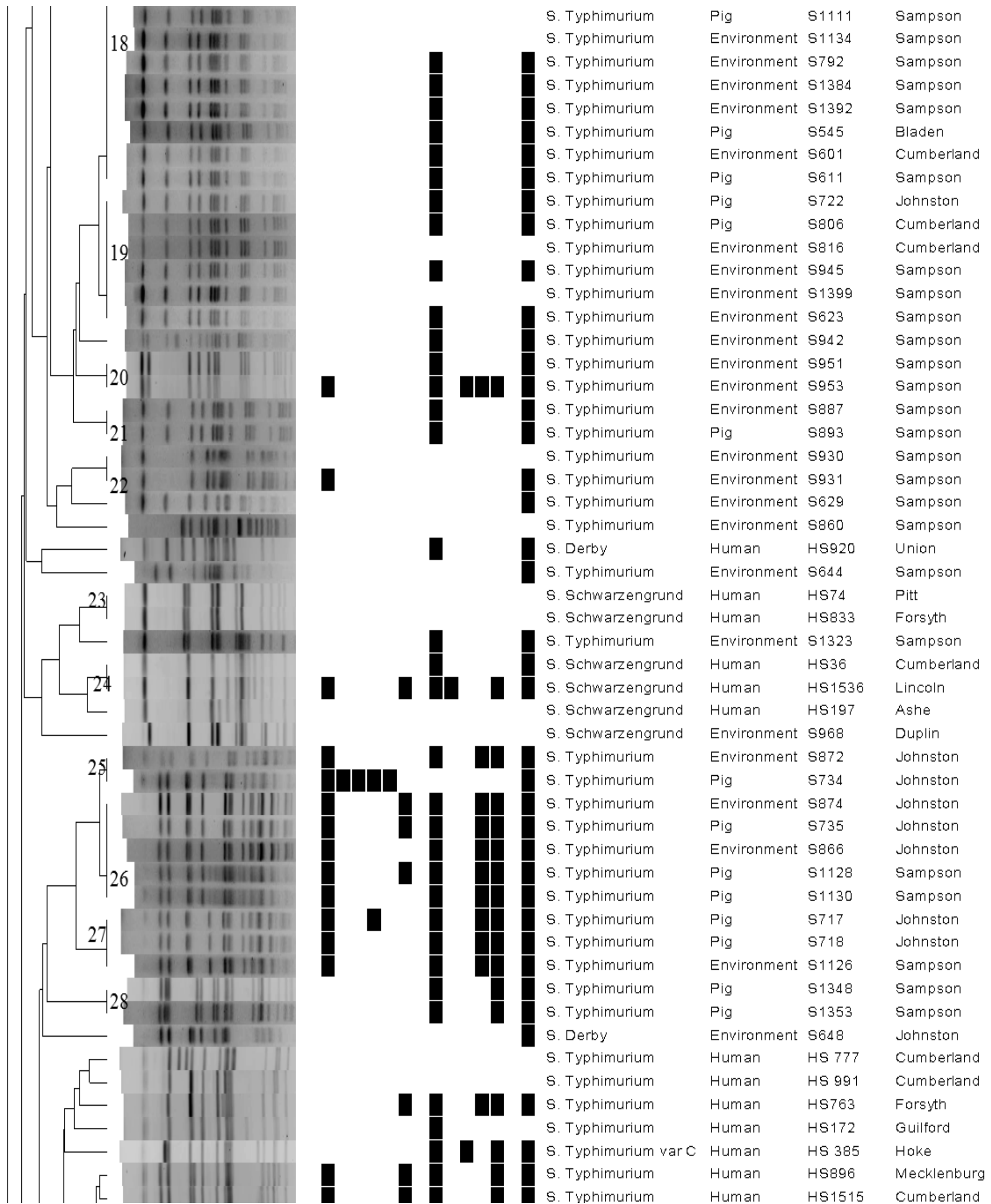
[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

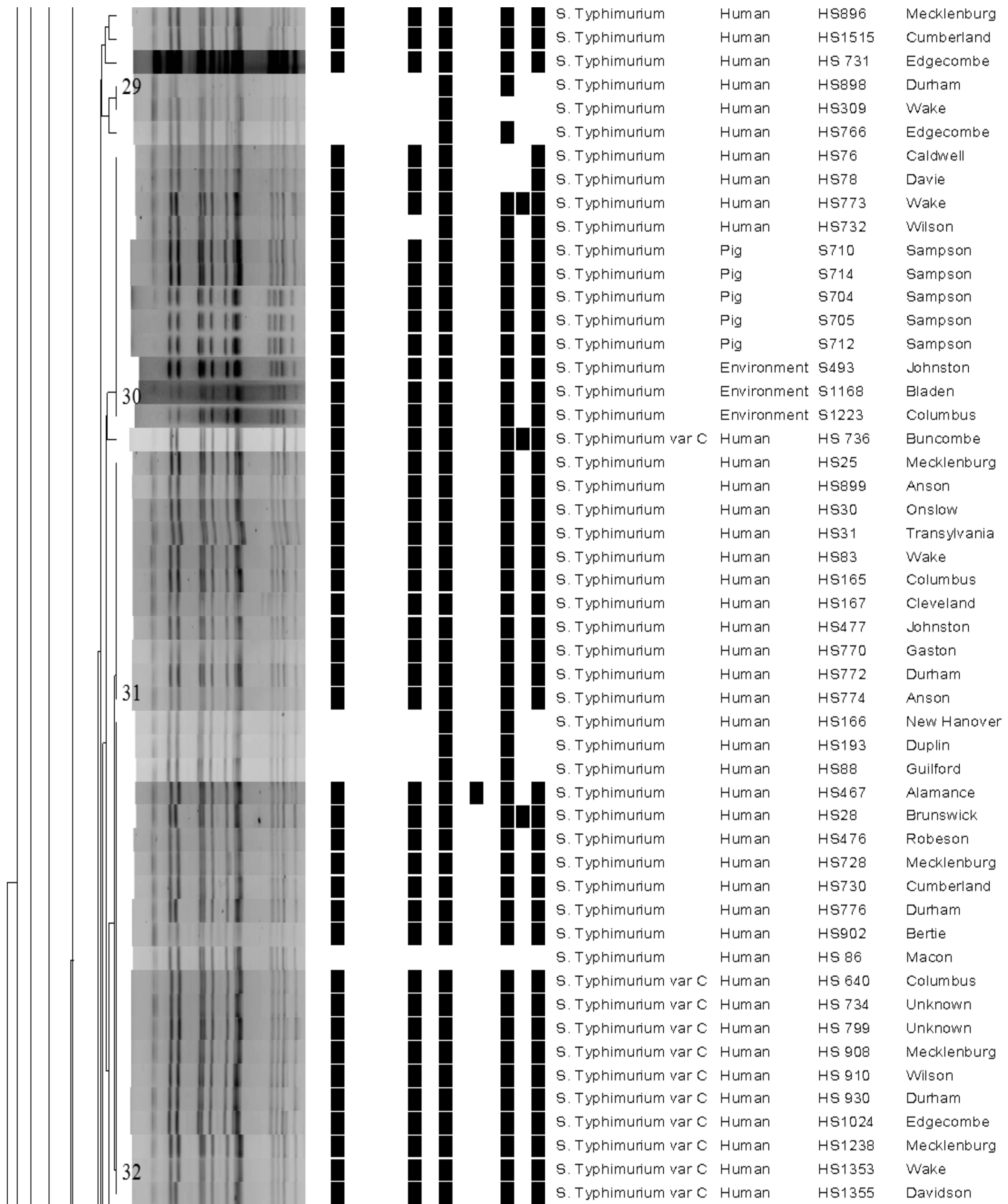
Figure 3.4 Dendrogram showing genotypic similarity among *Salmonella* serotypes isolated from humans, pigs and environment.

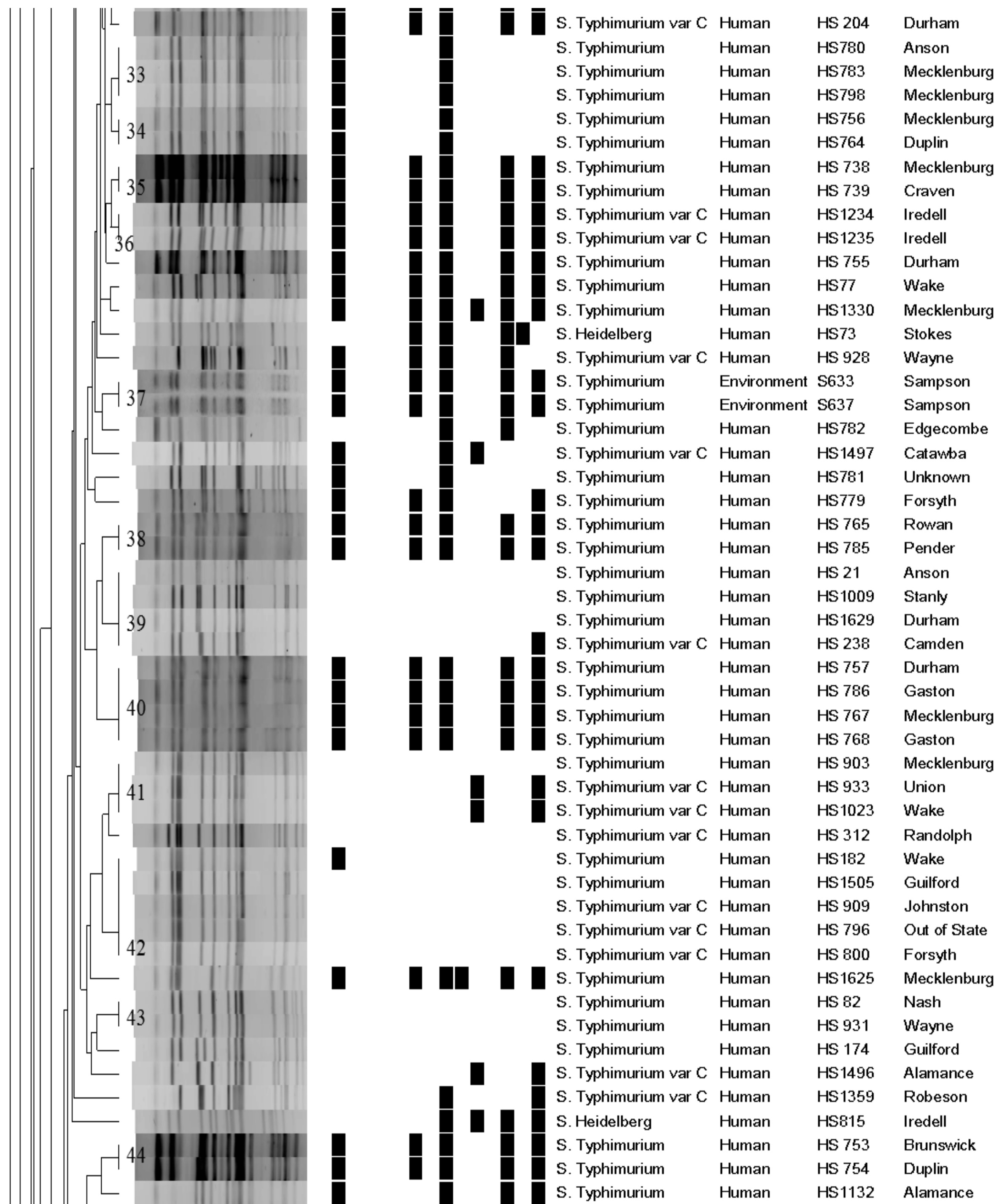
[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

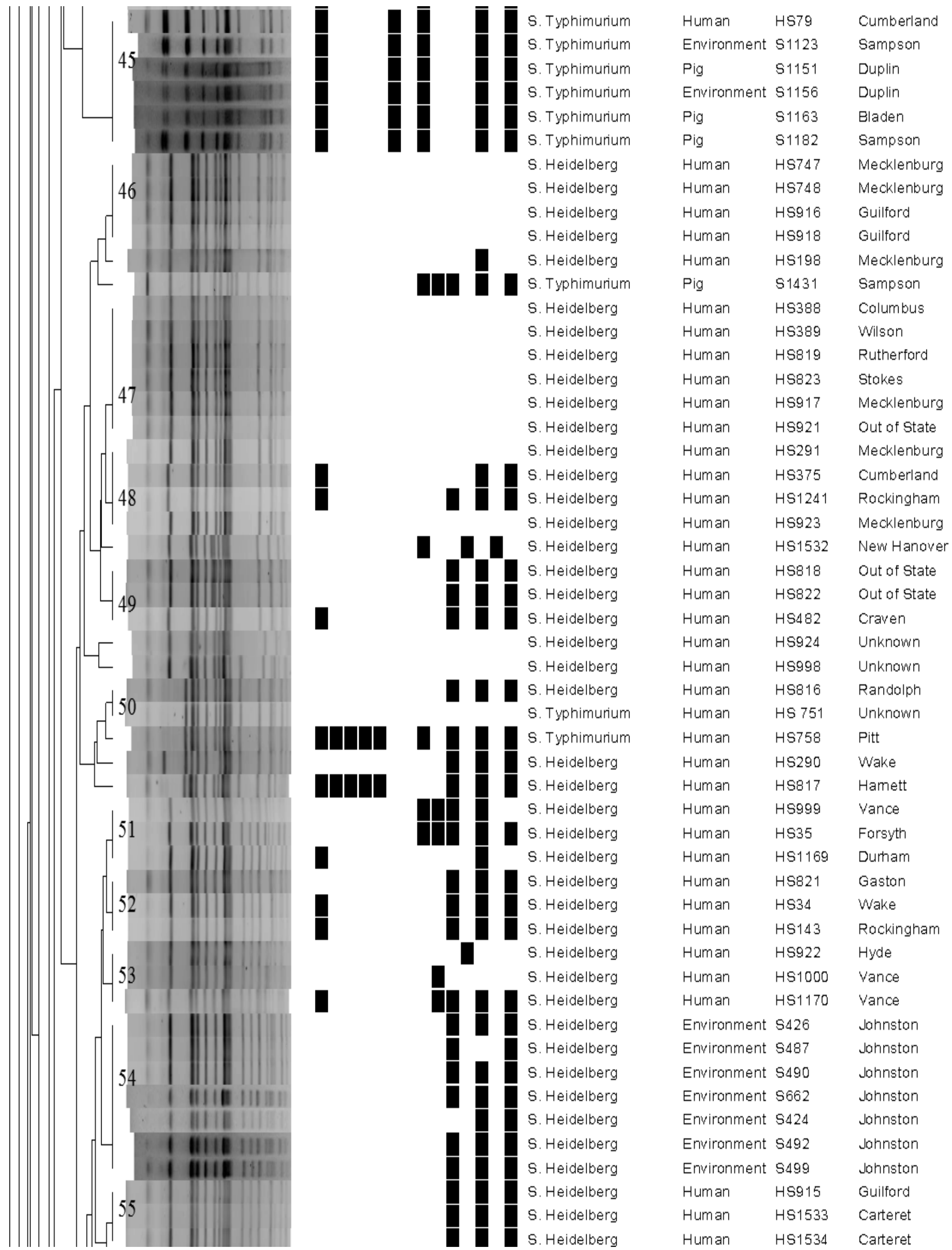

% Similarity
PFGE profile
AR profile[a]
Serotype
Source
ID
County
AMI
AMP
AUG
AXO
FOX
TIO
CHL
CIP
FIS
GEN
KAN
NAL
STR
SXT
TET
1
2
3
4
5
6
7
8
9
10
11
S. Typhimurium Environment S756 Johnston
S. Typhimurium Environment S1356 Sampson
S. Typhimurium Pig S666 Sampson
S. Heidelberg Human HS32 Wake
S. Derby Human HS1001 Pender
S. Derby Human HS1003 Pender
S. Derby Human HS919 Anson
S. Derby Human HS749 Sampson
S. Derby Human HS832 Anson
S. Derby Human HS1171 Pitt
S. Derby Environment S1013 Cumberland
S. Derby Pig S1023 Sampson
S. Derby Pig S1039 Sampson
S. Derby Pig S1044 Sampson
S. Derby Environment S329 Johnston
S. Derby Environment S324 Johnston
S. Derby Pig S427 Johnston
S. Derby Environment S502 Johnston
S. Derby Environment S1313 Sampson
S. Derby Environment S432 Johnston
S. Derby Environment S1010 Cumberland
S. Derby Pig S1048 Sampson
S. Derby Environment S1018 Cumberland
S. Derby Pig S1034 Sampson
S. Derby Pig S1019 Sampson
S. Derby Pig S1082 Sampson
S. Derby Pig S1085 Sampson
S. Derby Pig S1102 Sampson
S. Derby Human HS1002 Rutherford
S. Derby Environment S651 Johnston
S. Derby Pig S672 Sampson
S. Derby Environment S657 Johnston
S. Derby Environment S654 Johnston
S. Derby Human HS288 Carteret
S. Derby Human HS750 Unknown
S. Derby Human HS289 Cumberland
S. Derby Human HS75 Duplin
S. Muenchen Human HS292 Pender
S. Muenchen Human HS374 Pender
S. Muenchen Human HS49 Cabarrus
S. Muenchen Human HS51 Cabarrus
S. Muenchen Human HS54 Cabarrus
S. Muenchen Human HS192 Cabarrus
S. Muenchen Human HS190 Union
S. Muenchen Human HS1529 Onslow
S. Muenchen Human HS294 Mecklenburg
S. Muenchen Human HS144 Burke
S. Muenchen Human HS293 Montgomery

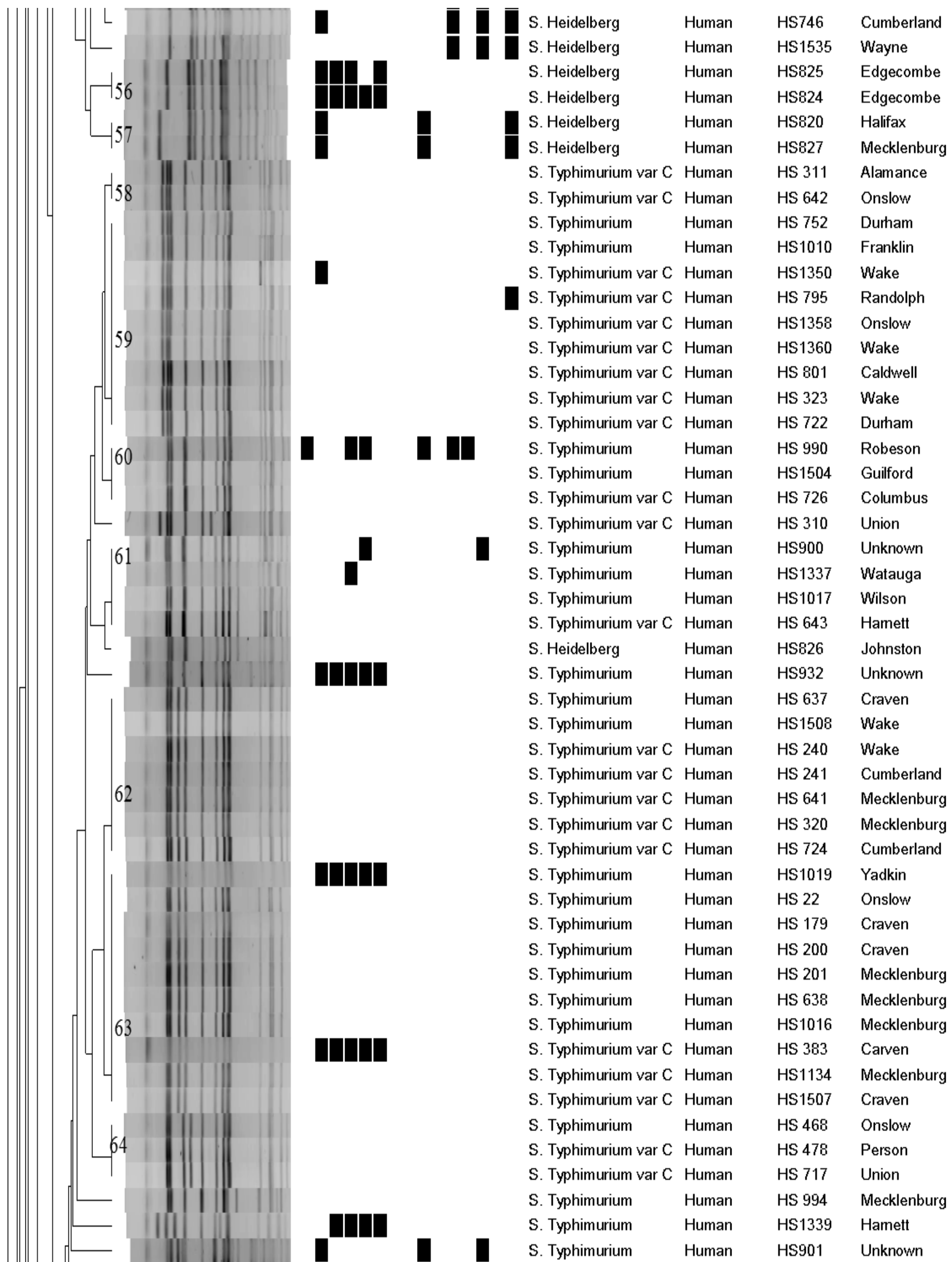

12
13
14
15
16
17
S. Muenchen Human HS191 Columbus
S. Muenchen Human HS1528 Duplin
S. Muenchen Human HS1530 Wayne
S. Muenchen Human HS645 New Hanover
S. Muenchen Human HS646 Unknown
S. Muenchen Human HS828 New Hanover
S. Muenchen Human HS1366 Alamance
S. Muenchen Human HS1362 Robeson
S. Muenchen Human HS480 Mecklenburg
S. Muenchen Human HS1006 Onslow
S. Muenchen Human HS45 Rutherford
S. Muenchen Human HS1168 Brunswick
S. Muenchen Human HS1365 Mecklenburg
S. Muenchen Human HS370 Brunswick
S. Muenchen Human HS1524 Sampson
S. Muenchen Human HS194 Wake
S. Muenchen Human HS37 Unknown
S. Muenchen Human HS43 Forsyth
S. Muenchen Human HS46 Forsyth
S. Muenchen Human HS148 Columbus
S. Muenchen Human HS743 Forsyth
S. Muenchen Human HS295 Duplin
S. Muenchen Human HS1004 Out of State
S. Muenchen Pig S478 Johnston
S. Muenchen Human HS296 New Hanover
S. Muenchen Human HS297 Cumberland
S. Muenchen Human HS371 Wake
S. Muenchen Human HS1526 Moore
S. Typhimurium Environment S938 Sampson
S. Muenchen Human HS39 Cumberland
S. Muenchen Human HS298 Wake
S. Schwarzengrund Environment S967 Duplin
S. Typhimurium Human HS897 Wake
S. Typhimurium Human HS904 Mecklenburg
S. Muenchen Human HS1166 Moore
S. Muenchen Human HS1167 Craven
S. Typhimurium Pig S800 Cumberland
S. Typhimurium Environment S932 Sampson
S. Heidelberg Human HS72 Henderson
S. Typhimurium Environment S496 Johnston
S. Typhimurium Human HS378 Guilford
S. Typhimurium Human HS180 Sampson
S. Typhimurium Pig S1176 Sampson
S. Heidelberg Environment S429 Johnston
S. Derby Environment S430 Johnston
S. Heidelberg Environment S607 Cumberland
S. Typhimurium var C Human HS 318 Mecklenburg
S. Typhimurium Environment S481 Johnston
S. Muenchen Human HS479 Lincoln
S. Muenchen Human HS1005 Rutherford
S. Typhimurium Pig S1110 Sampson
S. Typhimurium Pig S1111 Sampson

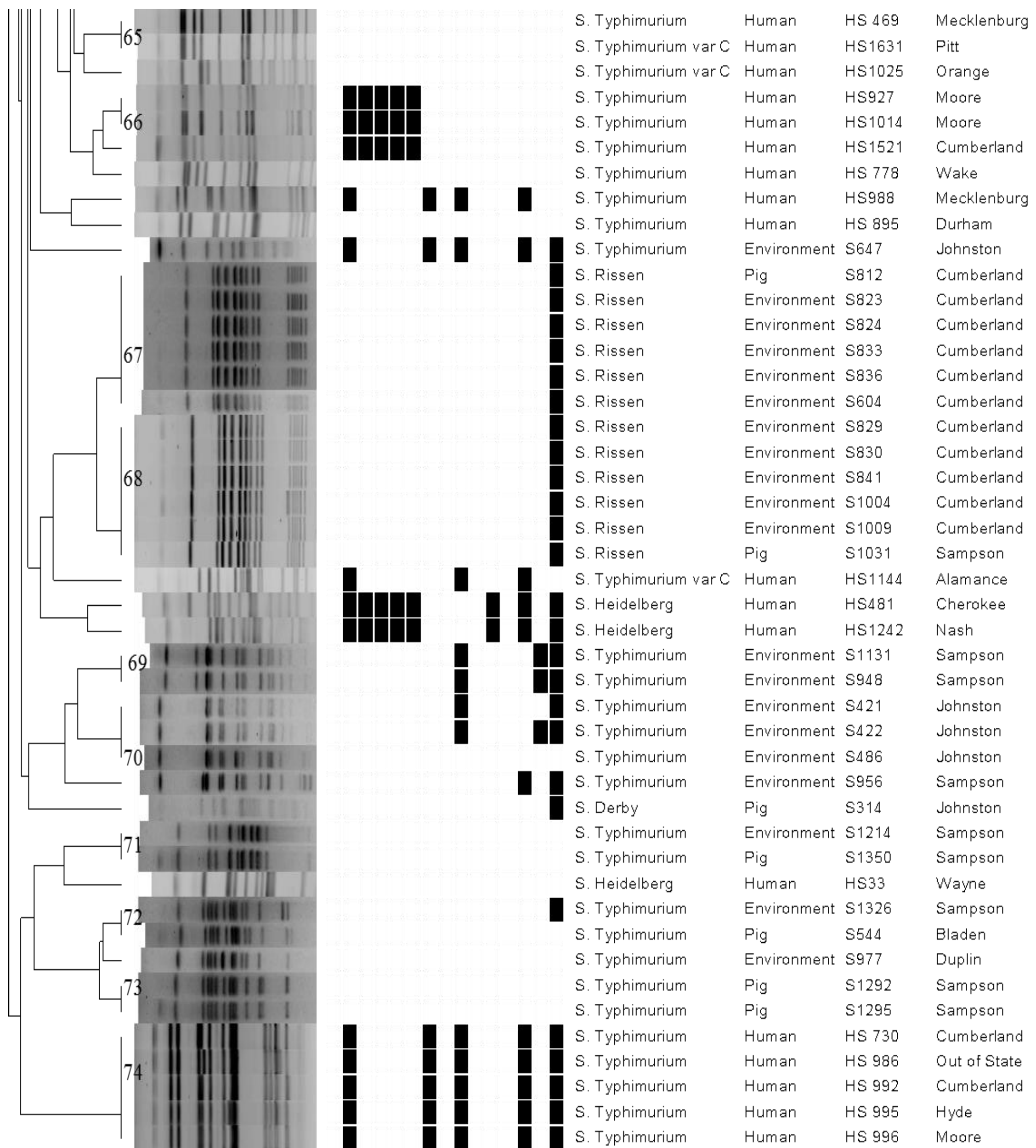

18
19
20
21
22
23
24
25
26
27
28
S. Typhimurium Pig S1111 Sampson
S. Typhimurium Environment S1134 Sampson
S. Typhimurium Environment S792 Sampson
S. Typhimurium Environment S1384 Sampson
S. Typhimurium Environment S1392 Sampson
S. Typhimurium Pig S545 Bladen
S. Typhimurium Environment S601 Cumberland
S. Typhimurium Pig S611 Sampson
S. Typhimurium Pig S722 Johnston
S. Typhimurium Pig S806 Cumberland
S. Typhimurium Environment S816 Cumberland
S. Typhimurium Environment S945 Sampson
S. Typhimurium Environment S1399 Sampson
S. Typhimurium Environment S623 Sampson
S. Typhimurium Environment S942 Sampson
S. Typhimurium Environment S951 Sampson
S. Typhimurium Environment S953 Sampson
S. Typhimurium Environment S887 Sampson
S. Typhimurium Pig S893 Sampson
S. Typhimurium Environment S930 Sampson
S. Typhimurium Environment S931 Sampson
S. Typhimurium Environment S629 Sampson
S. Typhimurium Environment S860 Sampson
S. Derby Human HS920 Union
S. Typhimurium Environment S644 Sampson
S. Schwarzengrund Human HS74 Pitt
S. Schwarzengrund Human HS833 Forsyth
S. Typhimurium Environment S1323 Sampson
S. Schwarzengrund Human HS36 Cumberland
S. Schwarzengrund Human HS1536 Lincoln
S. Schwarzengrund Human HS197 Ashe
S. Schwarzengrund Environment S968 Duplin
S. Typhimurium Environment S872 Johnston
S. Typhimurium Pig S734 Johnston
S. Typhimurium Environment S874 Johnston
S. Typhimurium Pig S735 Johnston
S. Typhimurium Environment S866 Johnston
S. Typhimurium Pig S1128 Sampson
S. Typhimurium Pig S1130 Sampson
S. Typhimurium Pig S717 Johnston
S. Typhimurium Pig S718 Johnston
S. Typhimurium Environment S1126 Sampson
S. Typhimurium Pig S1348 Sampson
S. Typhimurium Pig S1353 Sampson
S. Derby Environment S648 Johnston
S. Typhimurium Human HS 777 Cumberland
S. Typhimurium Human HS 991 Cumberland
S. Typhimurium Human HS763 Forsyth
S. Typhimurium Human HS172 Guilford
S. Typhimurium var C Human HS 385 Hoke
S. Typhimurium Human HS896 Mecklenburg
S. Typhimurium Human HS1515 Cumberland


29
30
31
32
S. Typhimurium Human HS896 Mecklenburg
S. Typhimurium Human HS1515 Cumberland
S. Typhimurium Human HS 731 Edgecombe
S. Typhimurium Human HS898 Durham
S. Typhimurium Human HS309 Wake
S. Typhimurium Human HS766 Edgecombe
S. Typhimurium Human HS76 Caldwell
S. Typhimurium Human HS78 Davie
S. Typhimurium Human HS773 Wake
S. Typhimurium Human HS732 Wilson
S. Typhimurium Pig S710 Sampson
S. Typhimurium Pig S714 Sampson
S. Typhimurium Pig S704 Sampson
S. Typhimurium Pig S705 Sampson
S. Typhimurium Pig S712 Sampson
S. Typhimurium Environment S493 Johnston
S. Typhimurium Environment S1168 Bladen
S. Typhimurium Environment S1223 Columbus
S. Typhimurium var C Human HS 736 Buncombe
S. Typhimurium Human HS25 Mecklenburg
S. Typhimurium Human HS899 Anson
S. Typhimurium Human HS30 Onslow
S. Typhimurium Human HS31 Transylvania
S. Typhimurium Human HS83 Wake
S. Typhimurium Human HS165 Columbus
S. Typhimurium Human HS167 Cleveland
S. Typhimurium Human HS477 Johnston
S. Typhimurium Human HS770 Gaston
S. Typhimurium Human HS772 Durham
S. Typhimurium Human HS774 Anson
S. Typhimurium Human HS166 New Hanover
S. Typhimurium Human HS193 Duplin
S. Typhimurium Human HS88 Guilford
S. Typhimurium Human HS467 Alamance
S. Typhimurium Human HS28 Brunswick
S. Typhimurium Human HS476 Robeson
S. Typhimurium Human HS728 Mecklenburg
S. Typhimurium Human HS730 Cumberland
S. Typhimurium Human HS776 Durham
S. Typhimurium Human HS902 Bertie
S. Typhimurium Human HS 86 Macon
S. Typhimurium var C Human HS 640 Columbus
S. Typhimurium var C Human HS 734 Unknown
S. Typhimurium var C Human HS 799 Unknown
S. Typhimurium var C Human HS 908 Mecklenburg
S. Typhimurium var C Human HS 910 Wilson
S. Typhimurium var C Human HS 930 Durham
S. Typhimurium var C Human HS1024 Edgecombe
S. Typhimurium var C Human HS1238 Mecklenburg
S. Typhimurium var C Human HS1353 Wake
S. Typhimurium var C Human HS1355 Davidson


33
34
35
36
37
38
39
40
41
42
43
44
S. Typhimurium var C Human HS 204 Durham
S. Typhimurium Human HS780 Anson
S. Typhimurium Human HS783 Mecklenburg
S. Typhimurium Human HS798 Mecklenburg
S. Typhimurium Human HS756 Mecklenburg
S. Typhimurium Human HS764 Duplin
S. Typhimurium Human HS 738 Mecklenburg
S. Typhimurium Human HS 739 Craven
S. Typhimurium var C Human HS1234 Iredell
S. Typhimurium var C Human HS1235 Iredell
S. Typhimurium Human HS 755 Durham
S. Typhimurium Human HS77 Wake
S. Typhimurium Human HS1330 Mecklenburg
S. Heidelberg Human HS73 Stokes
S. Typhimurium var C Human HS 928 Wayne
S. Typhimurium Environment S633 Sampson
S. Typhimurium Environment S637 Sampson
S. Typhimurium Human HS782 Edgecombe
S. Typhimurium var C Human HS1497 Catawba
S. Typhimurium Human HS781 Unknown
S. Typhimurium Human HS779 Forsyth
S. Typhimurium Human HS 765 Rowan
S. Typhimurium Human HS 785 Pender
S. Typhimurium Human HS 21 Anson
S. Typhimurium Human HS1009 Stanly
S. Typhimurium Human HS1629 Durham
S. Typhimurium var C Human HS 238 Camden
S. Typhimurium Human HS 757 Durham
S. Typhimurium Human HS 786 Gaston
S. Typhimurium Human HS 767 Mecklenburg
S. Typhimurium Human HS 768 Gaston
S. Typhimurium Human HS 903 Mecklenburg
S. Typhimurium var C Human HS 933 Union
S. Typhimurium var C Human HS1023 Wake
S. Typhimurium var C Human HS 312 Randolph
S. Typhimurium Human HS182 Wake
S. Typhimurium Human HS1505 Guilford
S. Typhimurium var C Human HS 909 Johnston
S. Typhimurium var C Human HS 796 Out of State
S. Typhimurium var C Human HS 800 Forsyth
S. Typhimurium Human HS1625 Mecklenburg
S. Typhimurium Human HS 82 Nash
S. Typhimurium Human HS 931 Wayne
S. Typhimurium Human HS 174 Guilford
S. Typhimurium var C Human HS1496 Alamance
S. Typhimurium var C Human HS1359 Robeson
S. Heidelberg Human HS815 Iredell
S. Typhimurium Human HS 753 Brunswick
S. Typhimurium Human HS 754 Duplin
S. Typhimurium Human HS1132 Alamance


45
46
47
48
49
50
51
52
53
54
55
S. Typhimurium Human HS79 Cumberland
S. Typhimurium Environment S1123 Sampson
S. Typhimurium Pig S1151 Duplin
S. Typhimurium Environment S1156 Duplin
S. Typhimurium Pig S1163 Bladen
S. Typhimurium Pig S1182 Sampson
S. Heidelberg Human HS747 Mecklenburg
S. Heidelberg Human HS748 Mecklenburg
S. Heidelberg Human HS916 Guilford
S. Heidelberg Human HS918 Guilford
S. Heidelberg Human HS198 Mecklenburg
S. Typhimurium Pig S1431 Sampson
S. Heidelberg Human HS388 Columbus
S. Heidelberg Human HS389 Wilson
S. Heidelberg Human HS819 Rutherford
S. Heidelberg Human HS823 Stokes
S. Heidelberg Human HS917 Mecklenburg
S. Heidelberg Human HS921 Out of State
S. Heidelberg Human HS291 Mecklenburg
S. Heidelberg Human HS375 Cumberland
S. Heidelberg Human HS1241 Rockingham
S. Heidelberg Human HS923 Mecklenburg
S. Heidelberg Human HS1532 New Hanover
S. Heidelberg Human HS818 Out of State
S. Heidelberg Human HS822 Out of State
S. Heidelberg Human HS482 Craven
S. Heidelberg Human HS924 Unknown
S. Heidelberg Human HS998 Unknown
S. Heidelberg Human HS816 Randolph
S. Typhimurium Human HS 751 Unknown
S. Typhimurium Human HS758 Pitt
S. Heidelberg Human HS290 Wake
S. Heidelberg Human HS817 Harnett
S. Heidelberg Human HS999 Vance
S. Heidelberg Human HS35 Forsyth
S. Heidelberg Human HS1169 Durham
S. Heidelberg Human HS821 Gaston
S. Heidelberg Human HS34 Wake
S. Heidelberg Human HS143 Rockingham
S. Heidelberg Human HS922 Hyde
S. Heidelberg Human HS1000 Vance
S. Heidelberg Human HS1170 Vance
S. Heidelberg Environment S426 Johnston
S. Heidelberg Environment S487 Johnston
S. Heidelberg Environment S490 Johnston
S. Heidelberg Environment S662 Johnston
S. Heidelberg Environment S424 Johnston
S. Heidelberg Environment S492 Johnston
S. Heidelberg Environment S499 Johnston
S. Heidelberg Human HS915 Guilford
S. Heidelberg Human HS1533 Carteret
S. Heidelberg Human HS1534 Carteret


56
57
58
59
60
61
62
63
64
S. Heidelberg Human HS746 Cumberland
S. Heidelberg Human HS1535 Wayne
S. Heidelberg Human HS825 Edgecombe
S. Heidelberg Human HS824 Edgecombe
S. Heidelberg Human HS820 Halifax
S. Heidelberg Human HS827 Mecklenburg
S. Typhimurium var C Human HS 311 Alamance
S. Typhimurium var C Human HS 642 Onslow
S. Typhimurium Human HS 752 Durham
S. Typhimurium Human HS1010 Franklin
S. Typhimurium var C Human HS1350 Wake
S. Typhimurium var C Human HS 795 Randolph
S. Typhimurium var C Human HS1358 Onslow
S. Typhimurium var C Human HS1360 Wake
S. Typhimurium var C Human HS 801 Caldwell
S. Typhimurium var C Human HS 323 Wake
S. Typhimurium var C Human HS 722 Durham
S. Typhimurium Human HS 990 Robeson
S. Typhimurium Human HS1504 Guilford
S. Typhimurium var C Human HS 726 Columbus
S. Typhimurium var C Human HS 310 Union
S. Typhimurium Human HS900 Unknown
S. Typhimurium Human HS1337 Watauga
S. Typhimurium Human HS1017 Wilson
S. Typhimurium var C Human HS 643 Harnett
S. Heidelberg Human HS826 Johnston
S. Typhimurium Human HS932 Unknown
S. Typhimurium Human HS 637 Craven
S. Typhimurium Human HS1508 Wake
S. Typhimurium var C Human HS 240 Wake
S. Typhimurium var C Human HS 241 Cumberland
S. Typhimurium var C Human HS 641 Mecklenburg
S. Typhimurium var C Human HS 320 Mecklenburg
S. Typhimurium var C Human HS 724 Cumberland
S. Typhimurium Human HS1019 Yadkin
S. Typhimurium Human HS 22 Onslow
S. Typhimurium Human HS 179 Craven
S. Typhimurium Human HS 200 Craven
S. Typhimurium Human HS 201 Mecklenburg
S. Typhimurium Human HS 638 Mecklenburg
S. Typhimurium Human HS1016 Mecklenburg
S. Typhimurium var C Human HS 383 Carven
S. Typhimurium var C Human HS1134 Mecklenburg
S. Typhimurium var C Human HS1507 Craven
S. Typhimurium Human HS 468 Onslow
S. Typhimurium var C Human HS 478 Person
S. Typhimurium var C Human HS 717 Union
S. Typhimurium Human HS 994 Mecklenburg
S. Typhimurium Human HS1339 Harnett
S. Typhimurium Human HS901 Unknown


65
66
67
68
69
70
71
72
73
74
S. Typhimurium Human HS 469 Mecklenburg
S. Typhimurium var C Human HS1631 Pitt
S. Typhimurium var C Human HS1025 Orange
S. Typhimurium Human HS927 Moore
S. Typhimurium Human HS1014 Moore
S. Typhimurium Human HS1521 Cumberland
S. Typhimurium Human HS 778 Wake
S. Typhimurium Human HS988 Mecklenburg
S. Typhimurium Human HS 895 Durham
S. Typhimurium Environment S647 Johnston
S. Rissen Pig S812 Cumberland
S. Rissen Environment S823 Cumberland
S. Rissen Environment S824 Cumberland
S. Rissen Environment S833 Cumberland
S. Rissen Environment S836 Cumberland
S. Rissen Environment S604 Cumberland
S. Rissen Environment S829 Cumberland
S. Rissen Environment S830 Cumberland
S. Rissen Environment S841 Cumberland
S. Rissen Environment S1004 Cumberland
S. Rissen Environment S1009 Cumberland
S. Rissen Pig S1031 Sampson
S. Typhimurium var C Human HS1144 Alamance
S. Heidelberg Human HS481 Cherokee
S. Heidelberg Human HS1242 Nash
S. Typhimurium Environment S1131 Sampson
S. Typhimurium Environment S948 Sampson
S. Typhimurium Environment S421 Johnston
S. Typhimurium Environment S422 Johnston
S. Typhimurium Environment S486 Johnston
S. Typhimurium Environment S956 Sampson
S. Derby Pig S314 Johnston
S. Typhimurium Environment S1214 Sampson
S. Typhimurium Pig S1350 Sampson
S. Heidelberg Human HS33 Wayne
S. Typhimurium Environment S1326 Sampson
S. Typhimurium Pig S544 Bladen
S. Typhimurium Environment S977 Duplin
S. Typhimurium Pig S1292 Sampson
S. Typhimurium Pig S1295 Sampson
S. Typhimurium Human HS 730 Cumberland
S. Typhimurium Human HS 986 Out of State
S. Typhimurium Human HS 992 Cumberland
S. Typhimurium Human HS 995 Hyde
S. Typhimurium Human HS 996 Moore

4. CHAPTER IV: Dissemination of identical trans-conjugal plasmids from antimicrobial resistant *Salmonella* serotypes originating from humans, pigs and their environment

Presented here is the manuscript titled "Dissemination of identical trans-conjugal plasmids from antimicrobial resistant Salmonella serotypes originating from humans, pigs and their environment", is submitted the present year (2013) and under peer review in the *Journal of Applied Microbiology*. Additional data are presented in the Appendix.

4.1 Abstract

Aim: The aim of this study was to characterize and determine the inter-serovar exchange of plasmids isolated from humans, pigs and their environment.

Methods and Results: A total of 21 antimicrobial resistant (AMR) *Salmonella* isolates representing human clinical cases (n=6), pigs (n=6) and the farm environment (n=9) were characterized. These isolates were part of longitudinal study conducted were earlier by our group representing multiple resistance patterns, serotypes, stages of swine production, and sources of origin. In the present study, these isolates were analyzed further for presence of plasmids, replicon typing, inter-serovar transferability by conjugation, and confirmation of AmpC β-lactamase enzyme encoding gene bla_{CMY-2} by southern hybridization. All the 21 AMR *Salmonella* isolates had conjugal plasmids ranging from 70-103kb in size and were able to transfer their resistance determinants to recipient *Escherichia coli* MG1655. Furthermore, the inter-serovar plasmid transfer was further confirmed by the PCR identification of the presence of respective resistance genes on the plasmid isolated from

trans-conjugants. The predominant replicon type of plasmid in the majority of study strains was I1-Iγ Inc group (17/21, 81%). However, the plasmids from the *S*. Rissen strains (91kb size plasmids) did not belong to any Inc group we tested in this study. Plasmids from *Salmonella* serovars Anatum, Ouakam, Johannesburg and Typhimurium isolated from the same cohort of pigs and their environment and *Salmonella* serovar Heidelberg from a single human clinical isolate had identical plasmid type and profile based (*EcoR*I, *Hind*III and *Pst*I restriction enzymes) and southern blotting.

Conclusions: There was clear evidence of inter-serovar exchange of resistance determinants through horizontal gene transfer among MDR *Salmonella* serotypes isolated from same cohort of pigs, environment within same farm and human clinical *Salmonella* isolates.

Significance and Impact of the Study: Occurrence of identical conjugal resistance plasmids of environmental origin and its inter-serovar exchange signify the crucial role of environment as a reservoir in persistence and dissemination of antimicrobial resistance determinants between humans and food animals.

4.2 Introduction

Salmonella enterica is one of the major zoonotic foodborne pathogens causing self-limiting gastrointestinal disease in humans and animals. *Salmonella* is a highly diverse pathogen with more than 2500 non-thyroidal *Salmonella* (NTS) serotypes, which are considered to be potential threat to humans (1). The occurrence and dissemination of antimicrobial resistant (AMR) *Salmonella* strains in humans, pigs and their environment which is attributed to sub-therapeutic use of antimicrobials for therapeutic and growth

purposes is under extensive debate in the US (2-4). Development of multidrug resistance (resistance to ≥ 3 antimicrobials; MDR) along with resistance to fluoroquinolones and cephalosporins is considered a significant public health concern since these drugs are critical in treating *Salmonella* infections and resistance can lead to treatment failure (5, 6). MDR *Salmonella* isolates, including those resistant to 3rd and 4th generation cephalosporins, associated with different *Salmonella* serotypes from humans, pigs, and their environment have been reported worldwide, including in the US (4, 7-10). These MDR isolates often carry resistance genes either on the chromosome or on mobile genetic elements such as plasmids and integrons (11).

Localization of resistance determinants on plasmids and horizontal gene transfer via conjugation results in rapid spread of AMR to susceptible bacterial populations (12). Plasmid mediated resistance has been identified in the majority of clinically important antimicrobials, including cephalosporins and quinolones (13-15). Plasmids carrying the $bla_{\text{CMY-2}}$ gene encoding resistance to multiple antimicrobials including ESBLs are predominant among *S*. Typhimurium, *S*. Heidelberg and *S*. Newport isolates (13). There are only a few studies that have demonstrated the exchange of plasmids within *Salmonella* serotypes isolated same farm and between human clinical isolates (8, 16). It is important to study the various factors associated with swine production as a reservoir for AMR determinants and its dissemination in food chain (13). Therefore, our study group conducted a longitudinal study to determine the dynamics of *Salmonella* in swine production system and their environment all along the production chain and compared with temporally and

spatially related predominant *Salmonella* serotypes from human clinical cases in the North Carolina.

We reported *Salmonella* prevalence, antimicrobial resistance profile, common MDR patterns, molecular characterization of resistance determinants and genotypic similarity among *Salmonella* isolates from humans, pigs and their environment at various stages of production (3, 4). Based on genotypic and phenotypic results, we were interested to determine whether different *Salmonella* serotypes with multiple resistance pattern (including MDR) harbor or exchange similar and/or different plasmids which carries multiple resistance determinants within temporally and spatially related MDR *Salmonella* serotypes in humans, pigs and their environment and to determine the possibility of horizontal gene transfer through plasmids. To address our hypothesis we isolated and characterized plasmids from different *Salmonella* serotypes representing three different sources, different/similar resistance patterns, and different stages of production at farm. We performed conjugation assays, plasmid restriction analysis and southern hybridization to identify conjugative plasmids and relatedness of plasmids isolated from these three different sources.

4.3 Materials and methods

4.3.1 Origin of *Salmonella* isolates

Salmonella isolates from pigs (n=386), environment (n=536) and human clinical cases (n=572) which are temporally and spatially related were isolated and characterized for their antimicrobial susceptibility, resistance determinants and fingerprint profile reported elsewhere (3, 4). The *Salmonella* isolates from pigs and their environment were collected as

part of longitudinal study conducted from October 2008 to December 2011 on 30 conventional farms at different stages of production from farm to slaughter in North Carolina. The details of the study design, sampling and microbiological methods, estimates of *Salmonella* prevalence in pigs and their environment at farm and slaughter, antimicrobial susceptibility profiles, and their phenotypic and genotypic characterizations have been reported elsewhere (3, 4). The conventional pigs were reared indoors and given antimicrobials for growth, prophylaxis and therapeutic purposes. The human clinical *Salmonella* isolates were temporally and spatially related and collected from North Carolina State Laboratory of Public Health (NCSLPH) during same period of longitudinal study period. From the above isolates, we selected a total of 21 MDR *Salmonella* isolates representing human clinical cases (n=6), pigs (n=6) and the environment (n=9). These isolates were representative of multiple resistance patterns, serotypes, stages of production and sources of origin were analyzed further for presence of plasmids and inter-serovar transferability in the present study.

4.3.2 Plasmid isolation and its characterization

The plasmids were isolated from the confirmed trans-conjugant (*E. coli*) cultures by an alkaline lysis method as described previously (17). The purified plasmid DNA concentration and its purity were determined using NanoDrop (Thermo Scientific, Waltham, Massachusetts, USA). Restriction fragment analysis of the plasmid was performed using the *EcoR*I, *Hind*III and *Pst*I enzyme with a restriction enzyme kit (Roche Diagnostics, Mannheim, Germany), according to manufacture guidelines.

The restricted plasmid DNA was electrophoresed in a horizontal 0.7% agarose gel in Tris-acetate buffer and gels were run at 15mA for 18h, along with molecular marker and photographed under UV light. Polymerase chain reaction (PCR) was done to confirm the resistance determinants carried on the plasmid using purified plasmid DNA from trans-conjugants as template DNA. The primers of confirmed AMR genes includes ampicillin resistance encoding genes bla_{TEM} and $bla_{PSE,}$ (18), ceftiofur and ceftriaxone resistance encoding gene bla_{CMY-2} genes (19), kanamycin resistance coding *aphAI* (20) and tetracycline resistance coding *tet(A)*, *tet(B)*, *tet(C)*, and *tet(G)* genes (21). Amplification reactions were carried out as described in the above studies.

4.3.3 Conjugation experiment, selection and confirmation of trans-conjugants

The MDR *Salmonella* isolates from humans, pigs and their environment represented in Table 4.1 were selected as donor strains and nalidixic acid (NAL) resistant *Escherichia coli* K-12 strain MG1655 was used as the recipient strain. The mating experiment was carried out on Luria Bertani (LB) plates (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) with specific antibiotics as a selective marker. The antibiotics and concentrations used in the selective plates were as follows: ampicillin (100µg/ml), ceftiofur (8µg/ml), ceftriaxone (10µg/ml), kanamycin (40µg/ml), nalidixic acid (50µg/ml) and tetracycline (20µg/ml). The protocol was carried out as described previously (22). Briefly, loop full of overnight grown donor and recipient strains were mixed on LB agar plates for mating and incubated at 37°C for 24h. The next day, the mixed culture was streaked on LB agar containing nalidixic acid (50µg/ml) and respective recipient selection antibiotic and incubated at 37°C for 24h.

Trans-conjugants were selected and re-streaked using tooth picks onto LB plates with antibiotics and *Salmonella* (Nontyphoidal) chromogenic plates (R & F, Downers Grove, IL, USA) for confirmation of trans-conjugant *E.coli*. The Donor and recipient strains with respective antibiotic were grown separately as a negative control. Further confirmation of AMR profile of trans-conjugants was performed by antimicrobial susceptibility testing against a panel of 15 antimicrobials using the broth micro-dilution method in a 96-well Sensititre™ plate CMV1AGNF (Trek Diagnostic Systems, Inc., Cleveland, OH). The panel of antimicrobials tested include: amikacin (AMI), gentamicin (GEN), kanamycin (KAN), streptomycin (STR), ampicillin (AMP), amoxicillin/clavulanic acid (AUG), ceftriaxone (AXO), cefoxitin (FOX), ceftiofur (TIO), ciprofloxacin (CIP), nalidixic acid (NAL), sulfisoxazole (FIS), trimethoprim/sulfamethaxazole (SXT), chloramphenicol (CHL), and tetracycline (TET). Testing procedure was carried out as described in the previous study (3). The MICs (minimum inhibitory concentrations) were recorded and breakpoints were determined based on CLSI recommendations (23).

4.3.4 Multiplex PCR for plasmid replicon typing

Plasmid replicon typing was completed to identify different types of plasmids. Eighteen pairs of primers were used in five multiplex and three single PCRs to identify FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons. The PCR reaction mixture and amplification was carried out as described previously (24). The purified plasmid DNA was used as template DNA. PCR running conditions used for amplifying the above replicons by multiplex PCR includes an initial denaturation for 5min at 94.8^{0}C and 30 cycles of denaturation for 1min at 95^{0}C, annealing for 30s at 60^{0}C, and

extension for 1min at 72^0C, with a final extension of 7 min at 72^0C. The single PCR reactions were performed with the same amplification conditions with an annealing temperature of 52.8^0C for 30s. The amplified products were analyzed by gel electrophoresis.

4.3.5 Southern hybridization

Southern blot analysis was done in order to confirm the relatedness of plasmids from *Salmonella* serotypes of three different sources of origin by hybridizing bla_{CMY-2} gene, which encodes resistance to ceftiofur/ceftriaxone. Representative isolates selected for the southern hybridization include *Salmonella* serovar Anatum (pig origin, S595/pS01, AMR pattern AMP AUG AXO FOX TIO TET), *Salmonella* serovar Anatum (pig origin, S732/pS02, AMR pattern AMP AUG AXO FOX TIO TET), *Salmonella* serovar Ouakam (environment origin, S770/pS03, AMR pattern AMP AUG AXO FOX TIO TET FIS), *Salmonella* serovar Typhimurium (environment origin, S773/pS04, AMR pattern AMP AUG AXO FOX TIO TET), *Salmonella* serovar Johannesburg (environment origin, S782/pS05, AMR pattern AMP AUG AXO FOX TIO TET), *Salmonella* serovar Heidelberg (human origin, HS1242/pHS01, AMR pattern AMP AUG AXO FOX TIO TET KAN STR). The *Salmonella* isolates from pigs and the environment were isolated from same cohort of pigs at farrowing and nursery stages (Table 4.1). The protocol was carried out as prescribed previously (16, 25). Briefly, purified plasmid DNA was digested with a restriction enzyme (*EcoRI*) for 4h and separated by gel electrophoresis on 1% agarose gel in 1X TAE (Tris-acetate-EDTA) buffer. Blotting was done by transferring restricted plasmid DNA onto a positively charged nylon membrane (Roche Diagnostics, Indianapolis, IN, USA) in transfer buffer with capillary action overnight at room temperature. After blotting, DNA on the nylon membrane was immobilized by UV

cross-linking for 2 min in a Stratalinker apparatus (Stratagene, La Jolla, California, USA). A digoxigenin (DIG) labeled *bla*$_{CMY-2}$ specific detection probe was generated using random primers according to the manufactures protocol. Pre-hybridization using DIG Easy Hyb solution followed and hybridization of the membrane with the denatured DIG labeled probe was carried out at 50^{0}C overnight with gentle agitation. After hybridization, the DIG labeled probe that was bound to the membrane was detected by chemiluminescence with anti-DIG alkaline phosphate and substrate after incubation at 37^{0}C for 10 min.

4.4 Results

4.4.1 Plasmid isolation and its characterization

Plasmids were isolated from all 21 AMR *Salmonella* isolates by alkaline lysis method. The plasmids were restricted with the *EcoR*I enzyme to determine the size and relatedness of plasmids based on restriction profiles with different serotypes and AMR profile. Human clinical *Salmonella* serotypes including *S.* Muenchen (HS295/pHS03) and *S.*Tyhpimurium (HS383/pHS04; HS1019/pHS05; HS758/pHS06), which have a similar MDR pattern (AMP AUG AXO FOX TIO), had different plasmid profiles based on restriction analysis. The estimated size of the plasmids from these isolates was between 80-100kb (Figure 4.1). Similarly, plasmids from TET resistant *S*. Rissen of environmental origin had a different restriction profile and with 91kb size plasmid (Figure 4.1).

Plasmids isolated from *Salmonella* serotypes belonging to the same cohort of pigs and their environment including *S.* Anatum (pig origin, S595/pS01 and S732/pS02, AMR pattern AMP AUG AXO FOX TIO TET), *S.* Ouakam (environment origin, S770/pS03, AMR pattern

AMP AUG AXO FOX TIO TET FIS), *S.* Typhimurium (environment origin, S773/pS04, AMR pattern AMP AUG AXO FOX TIO TET), *S.* Johannesburg (environment origin, S782/pS05, AMR pattern AMP AUG AXO FOX TIO TET) and human clinical *S.* Heidelberg strain(HS1242/pHS01, AMR pattern AMP AUG AXO FOX TIO TET KAN STR) were around 103kb size. The restriction analysis of these plasmids with the *EcoR*I enzyme exhibited a similar restriction profile, suggesting the presence of the same plasmid (Figure 4.2). Further analysis of these isolates with two additional restriction enzymes (*Hind*III and *Pst*I) confirmed the similar restriction profile, except *S*. Heidelberg from human origin which exhibited a different pattern when restricted with the *Hind*III enzyme (Figure 4.2). The remaining *Salmonella* isolates had different restriction profiles when restricted with the *EcoR*I enzyme and the approximate sizes of plasmids isolated from these serotypes are listed in Table 4.1. The presence of respective resistance genes on all the 21 AMR plasmids isolated in this study were detected by PCR including ampicillin resistance encoding genes bla_{TEM} and $bla_{\text{PSE,}}$ ceftiofur and ceftriaxone resistance encoding gene $bla_{\text{CMY-2}}$ genes, kanamycin resistance coding *aphAI* and tetracycline resistance coding *tet(A)* and *tet(C)*.

4.4.2 Conjugation experiment, selection and confirmation of trans-conjugants

The conjugation experiment was carried out to isolate and determine the presence of conjugative plasmids from MDR *Salmonella* strains representing humans, pigs and their environment. The MDR *Salmonella* isolates represented in Table 4.1 were selected as donor strains and nalidixic acid resistant *Escherichia coli* K-12 strain MG1655 was used as the recipient strain. All the 21 AMR *Salmonella* isolates from the three sources of origin had

conjugative plasmids and were isolated from confirmed trans-conjugants *E. coli* (Table 4.1). We also performed the same conjugation experiment to evaluate inter-serovar transmission of resistance determinants among *Salmonella* serotypes represented in Figure 4.2. *Salmonella* with MDR pattern AMP AUG AXO TIO TET (*S*. Anatum, n=4; *S*. Johannesburg, n=1and *S*. Typhimurium, n=1) were successfully transferred their plasmids to recipient *E*. coli and trans-conjugants were further selected on LB plates with nalidixic acid, ampicillin, ceftiofur and tetracycline antibiotics as the marker. Similarly, *Salmonella* serotypes with the MDR pattern AMP AUG AXO FOX TIO (*S*. Anatum, n=1; *S*. Muenchen, n=1 and *S*. Typhimurium, n=3) were selected on the above mentioned marker plates, without tetracycline. The plasmid from *S*. Rissen (TET resistant) isolated from trans-conjugants was selected on nalidixic acid and tetracycline marker LB plates. The antimicrobial susceptibility testing of trans-conjugants was conducted on 96 well-sensititre plates against respective antimicrobials and matched 100% with AMR profiles and MIC values of donor strains confirming effective transfer of plasmids from donor to recipient strains.

4.4.3 Multiplex PCR for plasmid replicon typing

A PCR based replicon typing consisting of five multiplex and three single PCRs reactions were carried out to identify different types of plasmids using previously published primers (24). All 21 AMR plasmids isolated from different *Salmonella* serotypes (Table 4.1) were screened. The predominant replicon type identified in our study was I1-Iγ Inc group (17/21, 81%), characterized by an amplicon size of 137bp (Figure 4.3). A plasmid from *S*. Typhimurium serotype with the AMR pattern AMP FIS NAL STR TET was typed as the

FIA Inc group with an amplicon size of 462bp (Figure 4.3). Three plasmids isolated from the *S*. Rissen serotype (TET Pattern) did not belong to any Inc group we tested in this study.

4.4.5 Southern hybridization

Southern blot analysis was carried out to confirm and compare the relatedness of plasmids of six *Salmonella* strains including a *S*. Heidelberg from a human clinical case (n=1), *S*. Anatum from pig fecal at farrowing and nursery stage, and *S*. Ouakam, *S*. Tyhpimurium, and *S*. Joannesburg from environment samples (lagoon) at the nursery stage. These *Salmonella* strains from pigs and the environment were isolated from same cohort (C5) at farrowing and nursery 1 production stage of conventional pigs. Southern hybridization was performed using a DIG labeled gene probe specific $bla_{\mathrm{CMY-2}}$ gene, which hybridized the gene on all the six *Salmonella* plasmids at the same location (4kb), confirming relatedness of plasmids as shown in Figure 4.4.

4.5 Discussion

Plasmids are considered as backbone for antimicrobial resistance determinants persistence and propagation in bacterial population. Identification and characterization of plasmids carrying multiple resistance determinants and its exchange among multiple serotypes humans, pigs and their environment is essential to understand the spread and persistence of antimicrobial resistance in the food chain and to mitigate the antimicrobial resistance problem. Therefore, the aim of this study is to characterize and determine the inter-serovar exchange of plasmids isolated from humans, pigs and their environment. In this study we selected 21 AMR *Salmonella* strains from humans, pigs and the environment that

were representative of identical and/or different phenotypic and genotypic characteristics. The AMR *Salmonella* strains characterized in this study were part of longitudinal study by our group involving temporally and spatially related *Salmonella* isolates from humans, pigs and their environment (3, 4). Briefly, the frequency of AMR and MDR was higher in *Salmonella* isolates of pig origin (AMR: 82.6%; MDR: 36%) followed by environmental (AMR: 67.6%; MDR: 21.4%) and human clinical isolates (AMR: 28%; MDR: 21.3%) (3, 4). We found identical resistance patterns, resistance determinants, and fingerprint profiles among *Salmonella* strains among the three sources. In addition, we detected the mobile genetic elements, Class I and Class II integrons in MDR *Salmonella* isolates, potentially suggesting their importance in the transmission of AR to susceptible population (3, 4).

We isolated plasmids from all 21 selected AMR *Salmonella* strains, resulting in plasmids ranging in size from 60 kb to 103 kb based on restriction analysis. These results support the previous studies reporting relatively larger plasmids from *Salmonella* strains (26-28). Previous reports have isolated plasmids from *S.* Muenchen and *S*. Typhimurium of human origin that exhibited a different plasmid profile even though they shared an identical MDR pattern (AMP AUG AXO FOX TIO), suggesting diversity of plasmids carrying same resistance determinants (29). However, we found a similar plasmid profile among *Salmonella* serotypes with the MDR pattern AMP AUG AXO FOX TIO TET of pig and environmental origin (Figure 4.1). We isolated plasmids from three *S*. Rissen isolates of environment origin that were resistant to a single antimicrobial (TET). The plasmid size from *S.* Rissen was 91kb, which was in contrast with a recent study reporting a 30kb plasmid from *S*. Rissen strains resistant to multiple antimicrobials, including tetracycline (30). To our

best knowledge this is first report of plasmid-mediated resistance in *S*. Rissen serotype from environmental origin in the United States. The *S*. Rissen is rarely found in the US and it was isolated from humans (< 20 isolates per year) from 1999 to 2007 and there were no reports of its occurrence in food animals in the US (31). It was reported to have entered the US in late 2008 and early 2009 through imported white pepper, resulting in a human outbreak in northern California and Nevada (32). This serotype is found to be rapidly disseminating in the environment (3). Identification of plasmid mediated resistance in this serotype alarms emergence of another AMR serotype associated with food animals and humans in the US. Further sequence analysis of this plasmid is necessary to determine the potential resistance determinants present on this plasmid.

Replicon typing by PCR of all the plasmids in our study typed predominantly as Inc gruop I1-Iγ encoding resistance to multiple antimicrobials including cephalosporins. The majority of the plasmids encoding resistance to cephalosporins (bla_{CMY-2} gene) identified in *Salmonella* in the US are categorized in the Inc A/C group and this plasmid is broadly disseminated among zoonotic foodborne pathogens (13, 19, 26, 33). However, in our study the AmpC β-lactamase encoding bla_{CMY-2} gene associated with Inc I1-Iγ plasmid suggests a diversified population of plasmids encoding resistance to the β-lactam group of antimicrobials.

All the plasmids isolated in this study were conjugal plasmids and have the potential to transfer resistance determinants among different *Salmonella* serotypes as reported previously (14, 16, 34). We evidenced inter-serovar exchange of resistance determinants among *Salmonella* strains including *S*. Anatum (pig origin) *S*. Typhimurium, *S*. Ouakam and

S. Johannesburg (environment origin) belonging to same cohort of pigs with multiple AMR patterns and a human clinical *S*. Heidelberg with a similar plasmid profile as reported previously (8, 16, 34). Identification of similar conjugal plasmids among different *Salmonella* serovars highlights the establishment of plasmids in different serotypes as their niche. In addition, plasmids of environmental origin and exchange between human and pig strains highlight the key role of environment as a link between them. These plasmids carried resistance determinants encoding for resistance to 3rd and 4th generation cephalosporins. PCR identification and confirmation of similar resistance determinants on these plasmids highlights the role of plasmids in the transmission of resistance determinants encoding resistance to critically important antimicrobials used in human, veterinary medicine leads to difficult for clinicians to treat infections and control spread of resistance determinants to susceptible bacterial populations (9, 27, 28). Southern hybridization of a DIG labeled bla_{CMY-2} gene on the plasmid confirmed the relatedness and inter-serovar exchange of identical plasmids among three different sources of origin as reported for kanamycin gene (16). In conclusion, detection of trans-conjugal plasmids in emerging and predominant *Salmonella* serotypes of human, pig and their environment is concerning since these serotypes are commonly associated with foodborne outbreaks. We identified and characterized identical conjugal plasmids responsible for inter-servar exchange of resistance determinants among humans, pigs and their environment highlighting other sources like environment as a reservoir for resistance determinants which contributes to rapid dissemination of antimicrobial resistance in food chain.

4.6 References

1. CDC. 2011. Foodborne Diseases Active Surveillance Network (FoodNet): Tables and Figures—2011 Preliminary Data. http://www.cdc.gov/foodnet/data/trends/tables/table5.html.

2. Dorr P, Tadesse D, Zewde B, Fry P, Thakur S, Gebreyes W. 2009. Longitudinal study of *Salmonella* dispersion and the role of environmental contamination in commercial swine production systems. Appl. Environ.Microbiol. 75:1478-1486.

3. Keelara S, Scott HM, Morrow WM, Gebreyes WA, Correa M, Nayak R, Stefanova R, Thakur S. 2013. Longitudinal study of distributions of similar antimicrobial-resistant *Salmonella* serovars in pigs and their environment in two distinct swine production systems. Appl.Environ. Microbiol. doi: 10.1128/AEM.01419-13.

4. Keelara S, Scott H, Morrow W, Hartley C, Griffin D, Gebreyes W, Thakur S. 2013. Comparative phenotypic and genotypic characterization of temporally and spatially related *Salmonella* isolated from Human clinical cases, Pigs and the Environment. Foodborne. Pathog. Dis. (under review).

5. Van Boxstael S, Dierick K, Van Huffel X, Uyttendaele M, Berkvens D, Herman L, Bertrand S, Wildemauwe C, Catry B, Butaye P, Imberechts H. 2012. Comparison of antimicrobial resistance patterns and phage types of *Salmonella* Typhimurium isolated from pigs, pork and humans in Belgium between 2001 and 2006. Food. Res. Int. 45:913-918.

6. Vo A, van Duijkeren E, Gaastra W, Fluit A. 2010. Antimicrobial resistance, class 1 integrons, and genomic island 1 in *Salmonella* isolates from Vietnam. Plos One. 5(2):e9440. doi: 10.1371/journal.pone.0009440.

7. Frye J, Fedorka-Cray P. 2007. Prevalence, distribution and characterisation of ceftiofur resistance in *Salmonella enterica* isolated from animals in the USA from 1999 to 2003. Int.J. Antimicrob. Agents 30:134-142.

8. Patchanee P, Zewde B, Taclesse D, Hoet A, Gebreyes W. 2008. Characterization of multidrug-resistant *Salmonella enterica* Serovar Heidelberg isolated from humans and animals. Foodborne. Pathog. Dis. 5:839-851.

9. Eller C, Simon S, Miller T, Frick JS, Prager R, Rabsch W, Guerra B, Werner G, Pfeifer Y. 2013. Presence of β-lactamases in extended-spectrum-cephalosporin-resistant *Salmonella enterica* of 30 different serovars in Germany 2005-11. J. Antimicrob. Chemother. May 14. [Epub ahead of print].

10. Glenn L, Lindsey R, Folster J, Pecic G, Boerlin P, Gilmour M, Harbottle H, Zhao S, McDermott P, Fedorka-Cray P, Frye J. 2013. Antimicrobial resistance genes in multidrug-resistant *Salmonella enterica* isolated from animals, retail meats, and humans in the United States and Canada. Microb. Drug. Resist. 19:175-184.

11. Aarestrup FM, Oliver Duran C, Burch DG. 2008. Antimicrobial resistance in swine production. Anim. Health. Res. Rev. 9:135-148.

12. Juhas M. 2013. Horizontal gene transfer in human pathogens. Crit Rev Microbiol. Jul 18. [Epub ahead of print].

13. Lindsey RL, Fedorka-Cray PJ, Frye JG, Meinersmann RJ. 2009. Inc A/C plasmids are prevalent in multidrug-resistant *Salmonella enterica* isolates. Appl. Environ. Microbiol. 75:1908-1915.

14. Akiyama T, Khan AA. 2012. Isolation and characterization of small qnrS1-carrying plasmids from imported seafood isolates of *Salmonella enterica* that are highly similar to plasmids of clinical isolates. FEMS. Immunol. Med. Microbiol. 64:429-432.

15. Seiffert SN, Hilty M, Perreten V, Endimiani A. 2013. Extended-spectrum cephalosporin-resistant Gram-negative organisms in livestock: an emerging problem for human health? Drug. Resist. Updat. 16:22-45.

16. Gebreyes W, Thakur S. 2005. Multidrug-resistant *Salmonella enterica* serovar Muenchen from pigs and humans and potential interserovar transfer of antimicrobial resistance. Antimicrob.Agents Chemother. 49:503-511.

17. Sambrook J, Russell D. 2001. Preparation of plasmid DNA by alkaline lysis with SDS: minipreparation, p. 1.32–1.34. In Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

18. Carlson S, Bolton L, Briggs C, Hurd H, Sharma V, Fedorka-Cray P, Jones B. 1999. Detection of multiresistant *Salmonella* Typhimurium DT104 using multiplex and fluorogenic PCR. Mol. Cell. Prob. 13:213-222.

19. Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD. 2001. Identification and expression of cephamycinase

bla(CMY) genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. Antimicrob. Agents Chemother. 45:3647-3650.

20. Frana T, Carlson S, Griffith R. 2001. Relative distribution and conservation of genes encoding aminoglycoside-modifying enzymes in *Salmonella enterica* serotype Typhimurium phage type DT104. Appl. Environ.Microbiol. 67:445-448.

21. Ng L, Mulvey M, Martin I, Peters G, Johnson W. 1999. Genetic characterization of antimicrobial resistance in Canadian isolates of *Salmonella* serovar Typhimurium DT104. Antimicrob.Agents Chemother.43:3018-3021.

22. Gebreyes W, Altier C. 2002. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp *enterica* serovar Typhimurium isolates from swine. J. Clin.Microbiol. 40:2813-2822.

23. CLSI. 2010. Performance standards for antimicrobial susceptibility testing;Twentieth informational supplement, p. 40-45, M100-S20. CLSI, Wayne, PA.

24. Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ. 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods. 63:219-228.

25. Sandvang D, Jensen LB, Baggesen DL, Baloda SB. 2000. Persistence of a *Salmonella* enterica serotype Typhimurium clone in Danish pig production units and farmhouse environment studied by pulsed field gel electrophoresis (PFGE). FEMS. Microbiol. Lett. 187:21-25.

26. Giles WP, Benson AK, Olson ME, Hutkins RW, Whichard JM, Winokur PL, Fey PD. 2004. DNA sequence analysis of regions surrounding *bla*$_{CMY-2}$ from multiple *Salmonella* plasmid backbones. Antimicrob. Agents Chemother. 48:2845-2852.

27. Mataseje LF, Baudry PJ, Zhanel GG, Morck DW, Read RR, Louie M, Mulvey MR. 2010. Comparison of CMY-2 plasmids isolated from human, animal, and environmental Escherichia coli and Salmonella spp. from Canada. Diagn. Microbiol. Infect. Dis. 67:387-391.

28. Carattoli A. 2011. Plasmids in Gram negatives: molecular typing of resistance plasmids. Int. J. Med. Microbiol. 301:654-658.

29. Winokur PL, Brueggemann A, DeSalvo DL, Hoffmann L, Apley MD, Uhlenhopp EK, Pfaller MA, Doern GV. 2000. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob. Agents Chemother. 44:2777-2783.

30. Antunes P, Mourão J, Pestana N, Peixe L. 2011. Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. J. Antimicrob. Chemother 66:2028-2032.

31. CDC. 2009. *Salmonella* Annual summary tables. Centers for Disease Control and Prevention, Atlanta, GA.

32. MMWR. 2010. *Salmonella* Montevideo Infections Associated with Salami Products Made with Contaminated Imported Black and Red Pepper — United States, July 2009–April 2010, p. 1638-1671, vol. 59.

33. Mulvey MR, Susky E, McCracken M, Morck DW, Read RR. 2009. Similar cefoxitin-resistance plasmids circulating in *Escherichia coli* from human and animal sources. Vet. Microbiol. 134:279-287.

34. Moodley A, Guardabassi L. 2009. Transmission of IncN plasmids carrying *bla*$_{CTX\text{-}M\text{-}1}$ between commensal *Escherichia coli* in pigs and farm workers. Antimicrob. Agents Chemother. 53:1709-1711.

Table 4.1 MDR *Salmonella* serotypes from humans, pigs and their environment characterized for plasmids.

Serotype	Isolate/Plasmid ID[a]	Cohort[b]	Stage[c]	Soruce	Sample type[d]	Resistance pattern[e]	Plasmid size[f]
S. Anatum	S530/pS07	C3	FA	Environment	Lagoon	AMP AUG AXO FOX TIO FIS	80-100kb
	S595/pS01	C5	FA	Pig	Fecal	AMP AUG AXO FOX TIO TET	103 kb
	S732/pS02	C5	N 1	Pig	Fecal	AMP AUG AXO FOX TIO TET	103 kb
	S813/pS12	C3	N 2	Pig	Fecal	AMP AUG AXO FOX TIO TET	103 kb
	S814/pS16	C3	N 2	Pig	Fecal	AMP AUG AXO FOX TIO TET	103 kb
	S1090/pS08	C3	Slaughter	Environment	Lairage	AMP AUG AXO FOX TIO	103 kb
S. Heidelberg	S607/pS14	C3	N 1	Environment	Lagoon	AMP KAN TET	60-80 kb
S. Johannesburg	S782/pS05	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX TIO TET	103 kb
S. Ouakam	S770/pS03	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX FIS TIO TET	103 kb
S. Rissen	S1004/pS17	C3	F 2	Environment	Floor swab	TET	91 kb
	S1005/pS18	C3	F 2	Environment	Floor swab	TET	91 kb
	S1006/pS19	C3	F 2	Environment	Floor swab	TET	91 kb
S. Typhimurium	S737/pS09	C5	N 1	Pig	Fecal	AMP FIS NAL STR TET	60-90 kb
	S773/pS04	C5	N 1	Environment	Lagoon	AMP AUG AXO FOX TIO TET	103 kb
	S1128/pS20	C5	Slaughter	Pig	MLN	AMP CHL FIS NAL STR TET	60-80 kb
S. Heidelberg	HS1242/pHS01	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO TET KAN STR	103 kb
S. Muenchen	HS295/pHS03	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO	80-100kb
S. Typhimurium	HS383/pHS04	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO	80-100kb
	HS758/pHS06	N/A	N/A	Human	N/A	AMP AUG AXO FOX FIS TIO TET KAN STR	80-100kb
	HS1014/pHS02	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO	80-100kb
	HS1019/pHS05	N/A	N/A	Human	N/A	AMP AUG AXO FOX TIO	80-100kb

[a]S: *Salmonella*, pS: plasmid *Salmonella*; HS: human; pHS:plasmid human *Salmonella*

[b]C3 and C5: conventional pigs and their environment belongs to cohort 3 and cohort 5 respectively; N/A: not applicable

Table 4.1(continued)

[c]FA: farrowing; N 1: nursery 1; N 2: nursery 2; F 2: finishing 2; N/A: not applicable

[d]MLN: mesenteric lymphnode

[e]AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, FIS: Sulfisoxazole, FOX: Cefoxitin, KAN: Kanamycin, STR: Streptomycin, TET: Tetracycline, TIO: Ceftiofur.

[f]Plasmid size range: its approximate determination of plasmid size based on restriction profile

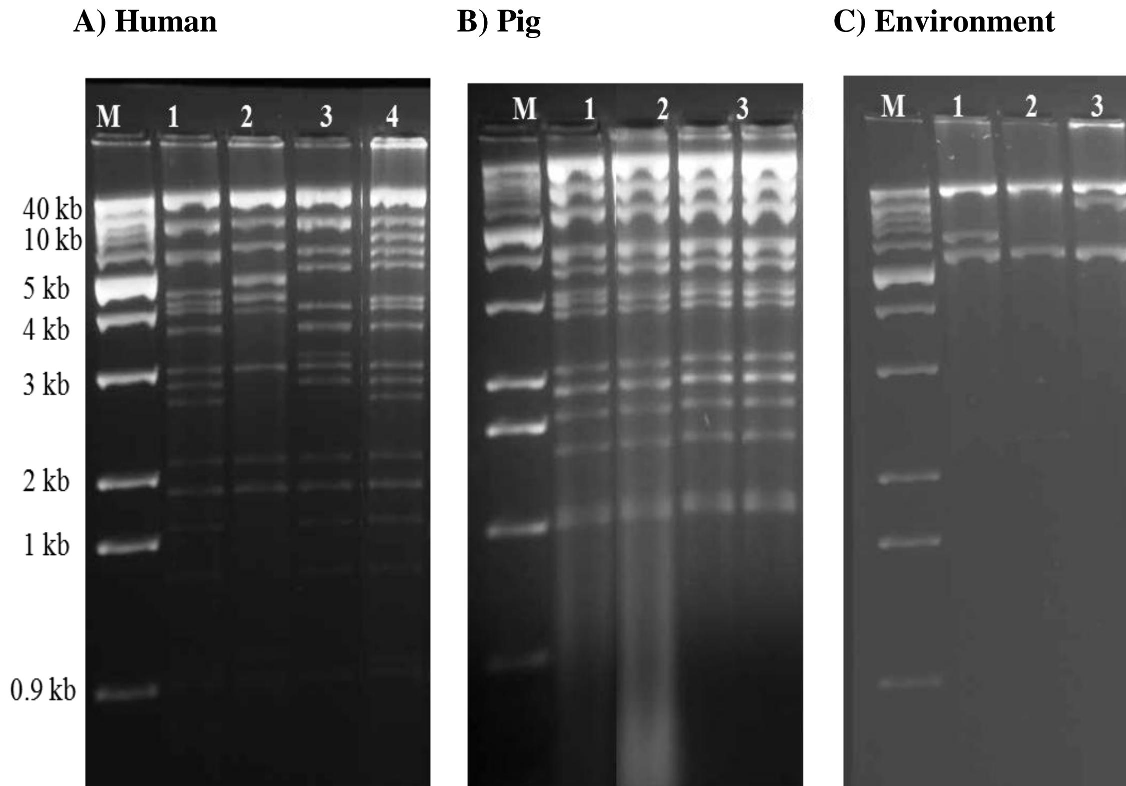
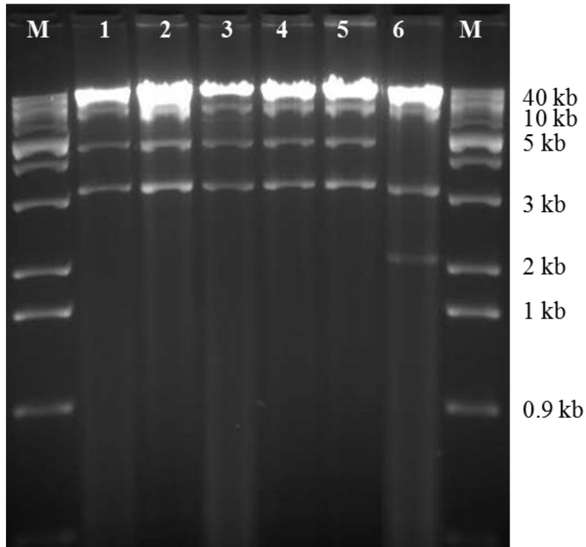
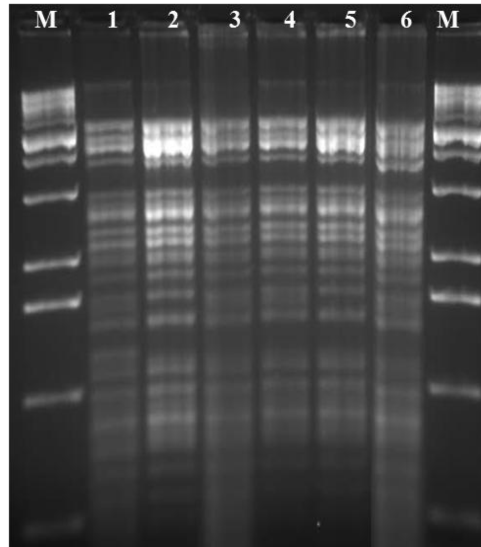


Figure 4.1 Plasmid restriction analysis with *EcoR*I enzyme of *Salmonella* serotypes.

A) Plasmid restriction analysis of *Salmonella* isolated from human clinical cases with AMR pattern AMP AUG AXO FOX TIO. Lane M: 40kb marker, lane 1, *S.* Muenchen (HS295/pHS03); 2, *S.* Tyhpimurium (HS383/pHS04); 3, *S.* Tyhpimurium (HS1019/pHS05); 4, *S.* Tyhpimurium (HS758/pHS06). B). Pig *Salmonella* isolates with AMR pattern C) Plasmids restriction analysis of *S.* Rissen isolated from environment which are resistant to TET (Lane 1, S1004/pS017; lane 2, S1005/pS018; lane 3, S1006/pS019).

A. *Hind*III **B. *Pst*I**





C. *EcoR*I

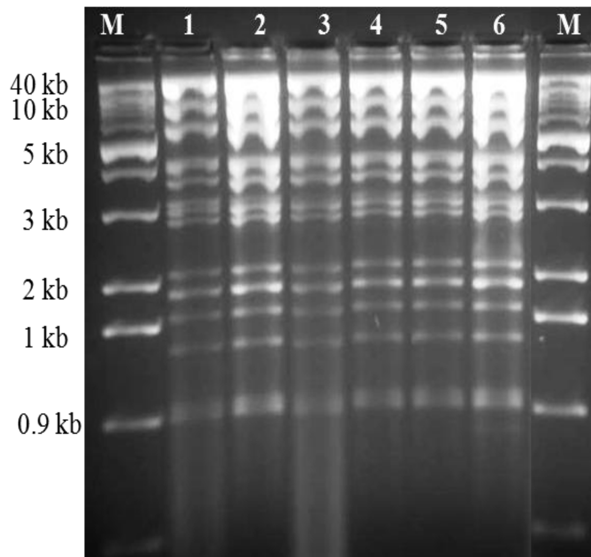
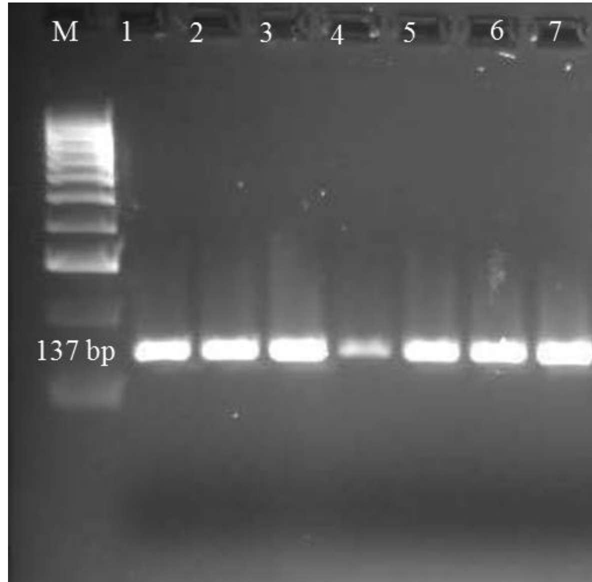


Figure 4.2 Plasmid restriction of β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and their environment.

Figure 4.2 (continued)

Plasmid restriction analysis of *Salmonella* isolated from humans, pig and their environment (belongs to same cohort and farm) with following restriction enzymes.A) *Hind*III; B) *Pst*I; C) *Eco*RI. Lanes M: 40kb marker; lane 1, *S.* Anatum (pig origin, S595/pS01, AMR pattern AMP AUG AXO FOX TIO TET); 2, *S.* Anatum (pig origin, S732/pS02, AMR pattern AMP AUG AXO FOX TIO TET); 3, *S.* Ouakam (environment origin, S770/pS03, AMR pattern AMP AUG AXO FOX TIO TET FIS); 4, *S.* Typhimurium (environment origin, S773/pS04, AMR pattern AMP AUG AXO FOX TIO TET); 5, *S.* Johannesburg (environment origin, S782/pS05, AMR pattern AMP AUG AXO FOX TIO TET); 6, *S.* Heidelberg (human origin, HS1242/pHS01, AMR pattern AMP AUG AXO FOX TIO TET KAN STR).

A) B)



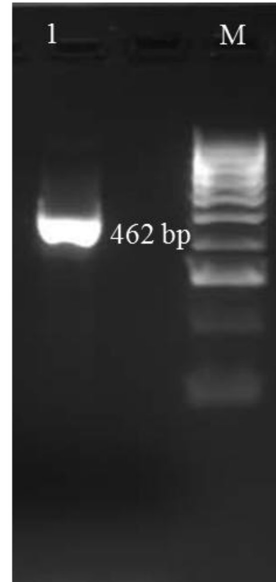


Figure 4.3 Multiplex PCR for plasmid replicon typing.

A) Lane M: 1kb DNA marker; lane 1-7: *Salmonella* serotypes amplifying 137 bp amplicon corresponding to Inc I1-Iγ plasmid type. B) Lane M: 1kb DNA marker; lane 1: S. Typhimurium plasmid with an amplicon size of 462bp corresponding to Inc FIA plasmid type.

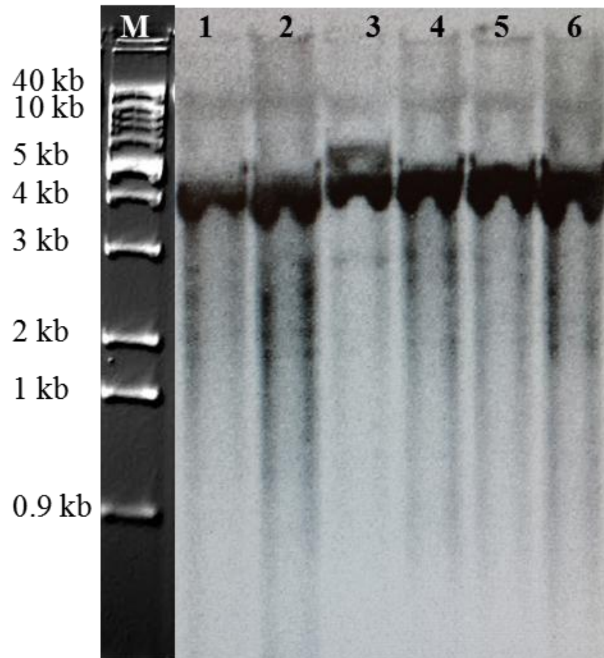


Figure 4.4 Southern hybridization of bla_{CMY-2} gene in β-lactam resistant MDR *Salmonella* serotypes from humans, pigs and their environment

Southern hybridization of all seven MDR *Salmonella* isolates with DIG labeled bla_{CMY-2} gene. Lanes M: 40kb marker; lane 1, *S.* Anatum (pig origin, S595/pS01, AMR pattern AMP AUG AXO FOX TIO TET); 2, *S.* Anatum (pig origin, S732/pS02, AMR pattern AMP AUG AXO FOX TIO TET); 3, *S.* Ouakam (environment origin, S770/pS03, AMR pattern AMP AUG AXO FOX TIO TET FIS); 4, *S.* Typhimurium (environment origin, S773/pS04, AMR pattern AMP AUG AXO FOX TIO TET); 5, *S.* Johannesburg (environment origin, S782/pS05, AMR pattern AMP AUG AXO FOX TIO TET); 6, *S.* Heidelberg (human origin, HS1242/pHS01, AMR pattern AMP AUG AXO FOX TIO TET KAN STR). All the serotypes irrespective of sources had the gene on the same restriction fragment, which was 4 kb.

5. APPENDICES

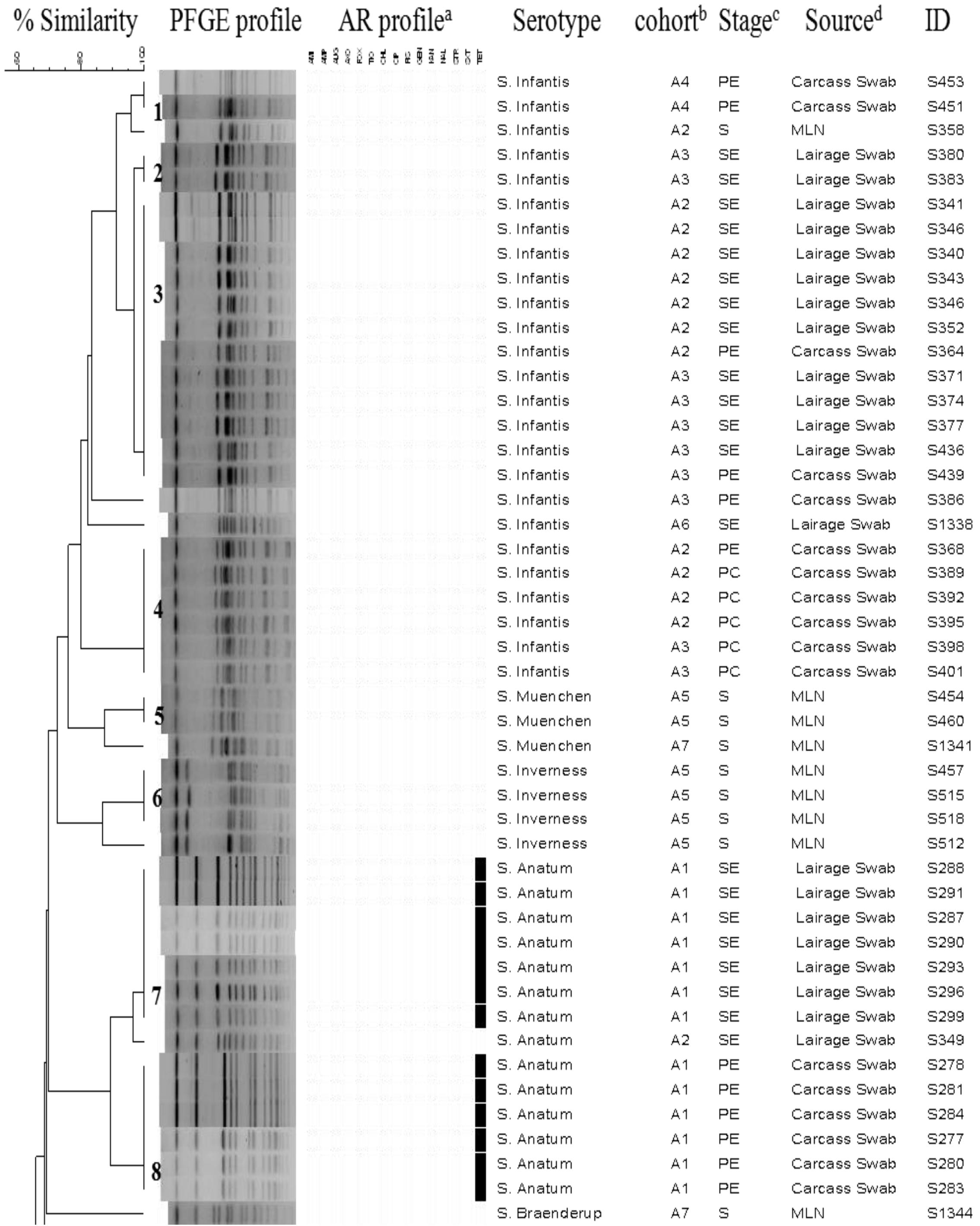
Appendix 5. 1 Dendrogram exhibiting genotypic similarity among *Salmonella* isolates from ABF production system at farm and slaughter

[a]AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur.

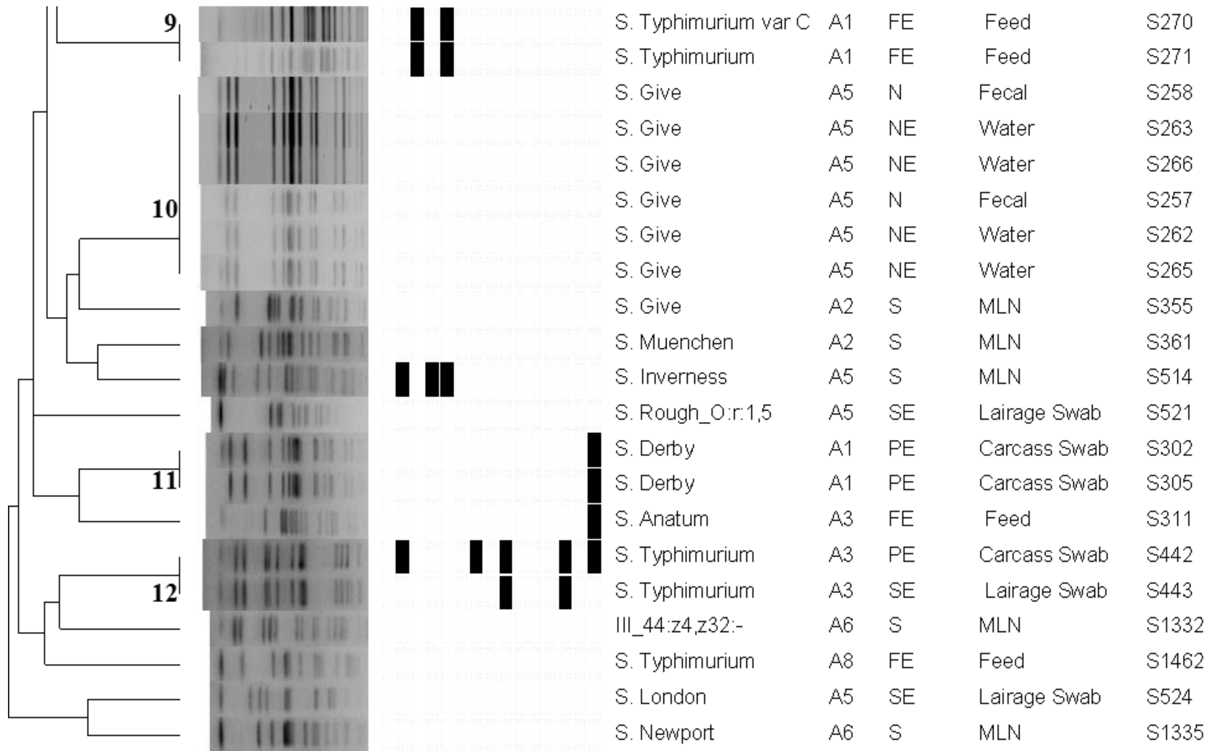
[b]A1-A8: ABF cohorts from 1 to 8

[c]PE: Post-evisceration, PC: Post-chill, S: Slaughter, SE: Slaughter environment, FE: Finishing environment, N: Nursery fecal, NE: Nursery environment,

[d]MLN: Mesenteric lymphnode.



% Similarity
PFGE profile
AR profile[a]
Serotype
cohort[b]
Stage[c]
Source[d]
ID
1
2
3
4
5
6
7
8
S. Infantis A4 PE Carcass Swab S453
S. Infantis A4 PE Carcass Swab S451
S. Infantis A2 S MLN S358
S. Infantis A3 SE Lairage Swab S380
S. Infantis A3 SE Lairage Swab S383
S. Infantis A2 SE Lairage Swab S341
S. Infantis A2 SE Lairage Swab S346
S. Infantis A2 SE Lairage Swab S340
S. Infantis A2 SE Lairage Swab S343
S. Infantis A2 SE Lairage Swab S346
S. Infantis A2 SE Lairage Swab S352
S. Infantis A2 PE Carcass Swab S364
S. Infantis A3 SE Lairage Swab S371
S. Infantis A3 SE Lairage Swab S374
S. Infantis A3 SE Lairage Swab S377
S. Infantis A3 SE Lairage Swab S436
S. Infantis A3 PE Carcass Swab S439
S. Infantis A3 PE Carcass Swab S386
S. Infantis A6 SE Lairage Swab S1338
S. Infantis A2 PE Carcass Swab S368
S. Infantis A2 PC Carcass Swab S389
S. Infantis A2 PC Carcass Swab S392
S. Infantis A2 PC Carcass Swab S395
S. Infantis A3 PC Carcass Swab S398
S. Infantis A3 PC Carcass Swab S401
S. Muenchen A5 S MLN S454
S. Muenchen A5 S MLN S460
S. Muenchen A7 S MLN S1341
S. Inverness A5 S MLN S457
S. Inverness A5 S MLN S515
S. Inverness A5 S MLN S518
S. Inverness A5 S MLN S512
S. Anatum A1 SE Lairage Swab S288
S. Anatum A1 SE Lairage Swab S291
S. Anatum A1 SE Lairage Swab S287
S. Anatum A1 SE Lairage Swab S290
S. Anatum A1 SE Lairage Swab S293
S. Anatum A1 SE Lairage Swab S296
S. Anatum A1 SE Lairage Swab S299
S. Anatum A2 SE Lairage Swab S349
S. Anatum A1 PE Carcass Swab S278
S. Anatum A1 PE Carcass Swab S281
S. Anatum A1 PE Carcass Swab S284
S. Anatum A1 PE Carcass Swab S277
S. Anatum A1 PE Carcass Swab S280
S. Anatum A1 PE Carcass Swab S283
S. Braenderup A7 S MLN S1344



9
10
11
12
S. Typhimurium var C A1 FE Feed S270
S. Typhimurium A1 FE Feed S271
S. Give A5 N Fecal S258
S. Give A5 NE Water S263
S. Give A5 NE Water S266
S. Give A5 N Fecal S257
S. Give A5 NE Water S262
S. Give A5 NE Water S265
S. Give A2 S MLN S355
S. Muenchen A2 S MLN S361
S. Inverness A5 S MLN S514
S. Rough_O:r:1,5 A5 SE Lairage Swab S521
S. Derby A1 PE Carcass Swab S302
S. Derby A1 PE Carcass Swab S305
S. Anatum A3 FE Feed S311
S. Typhimurium A3 PE Carcass Swab S442
S. Typhimurium A3 SE Lairage Swab S443
III_44:z4,z32:- A6 S MLN S1332
S. Typhimurium A8 FE Feed S1462
S. London A5 SE Lairage Swab S524
S. Newport A6 S MLN S1335

Appendix 5. 2 Overall *Salmonella* prevalence in ABF and conventional production systems.

Stage	Production System[a]	Source	Total no of Samples	No of Positive Samples	Prevalence (%)	Total no of Isolates (n)	Resistant (n)[b]	PanSusceptible (n)[c]	MDR (n)[d]
Farm	ABF	Pigs	1239	2	0.16	5	5	0	0
		Environment	797	5	0.62	14	5	9	0
	Conventional	Pigs	1650	66	4	189	155	34	35
		Environment	1325	156	11.7	439	390	49	98
Slaughter	ABF	Carcass	565	31	5.4	86	17	69	2
		Environment	115	21	18.2	63	21	42	0
	Conventional	Carcass	798	67	8.3	197	157	40	105
		Environment	90	35	38.8	97	36	61	17

[a] ABF: Antimicrobial free

[b] Resistant: *Salmonella* isolates exhibiting resistance to at least one antimicrobial.

[c] Pansusceptible: *Salmonella* isolates susceptible to all the antimicrobials tested.

[d] MDR: *Salmonella* isolates exhibiting resistance to ≥ 3 antimicrobials.

Appendix 5. 3 *Salmonella* prevalence in ABF and Conventional farms by production stage and sample type.

Farm									
Pig						Environment			
Sampling Stage	Farm Type[a]	Type of Samples	Proportion % (n/N)[b]	*P*	95% CI	Type of Samples[c]	Proportion % (n/N)	*P*	95% CI
Farrowing[d]	ABF	Fecal	0 (0/283)	N/A	N/A	W, F, S, FS,	0 (0/159)	N/A	N/A
	Conv	Fecal	6.7 (24/420)	0.001	0.260-0.108	W, F, S, FS, L	8.4 (21/250)	0.00 5	0.025-0.142
Nursery 1	ABF	Fecal	0.7 (1/247)	0.296	-0.006-0.021	W, F, S, FS,	1.2 (10/161)	0.29 6	-0.006-0.021
	Conv	Fecal	8.8 (17/333)	0.009	0.021-0.156	W, F, S, FS, L	13.5 (36/250)	0.00 2	0.049-0.222
Nursery 2	ABF	Fecal	0.2 (1/245)	0.296	-0.002-0.007	W, F, S, FS,	0 (0/160)	N/A	N/A
	Conv	Fecal	7.2 (10/318)	0.015	0.013-0.130	W, F, S, FS, L,T	12.4 (64/285)	0.00 7	0.338-0.214
Finishing 1	ABF	Fecal	0.2 (0/239)	0.297	-0.002-0.007	W, F, S, FS,	0.6 (1/157)	0.29 8	-0.006-0.021
	Conv	Fecal	6.7 (3/296)	0.002	0.024-0.110	W, F, S, FS, L	12.5 (33/250)	0.00 1	0.0481-0.203
Finishing 2	ABF	Fecal	0.5 (0/225)	0.114	-0.001-0.011	W, F, S, FS,	1.2 (2/160)	0.11 3	-0.006-0.021
	Conv	Fecal	7.7 (12/283)	0.005	0.022-0.132	W, F, S, FS, L,T	11.8 (33/290)	0.01 1	0.026-0.209

Appendix 5. 3 (continued)

[a] ABF: Antimicrobial free, Conv: conventional.

[b] The number of positive *Salmonella* samples/ the total number of samples is indicated in parentheses.

[c] W: water, F: feed, S: soil, FS: floor swabs, L: lagoon, T: truck swab.

[d] The farrowing stage results include sows in lactation and piglets as follows: sows (ABF, 0%, 0/39; conventional, 4.2%, 3/70) ($P = 0.11$) and piglets (ABF, 0%, 0/244; conventional, 6%, 21/350) ($P < 0.001$).

NA: not applicable.

Appendix 5. 4 *Salmonella* prevalence in ABF and conventional carcass and the environment at slaughter.

Source	Sample Type[a]	Production Type[b]	Prevalence % (n/N)[c]	*P*	95% CI
Carcass	MLN	ABF Conv	7.0 (13/184) 22.1 (60/271)	< 0.01	0.065-0.161
	Post Evisceration	ABF Conv	7.1 (13/182) 2.2 (6/272)	0.12	0.018-0.124
	Post Chill	ABF Conv	2.5 (5/199) 1.6 (4/255)	0.37	-0.005-0.055
Environment	Lairage	ABF Conv	26.2 (21/80) 46 (23/50)	0.23	0.085-0.279
	Slaughter truck	ABF Conv	0 (0/35) 30 (12/40)	0.05	0.265-0.512

[a] MLN: Mesenteric lymphnode.

[b]ABF: Antimicrobial free, Conv: conventional.

[c] The number of positive *Salmonella* samples/ the total number of samples is indicated in parenthes.

Appendix 5. 5 Minimum inhibitory concentration (MIC) levels and frequency of resistance of *Salmonella* isolates from ABF and conventional production systems.

Antimicrobial and parameter[a]	Farm						Slaughter					
	Pig			Environment			Carcass			Environment		
	ABF[a] n=5	Conv[b] n=189	*P*	ABF n=14	Conv n=439	*P*	ABF n=86	Conv n=197	*P*	ABF n=63	Conv n=97	*P*
AMI												
MIC $_{50}$	4	1		2	1		1	2		2	1	
MIC $_{90}$	4	2	0.22	4	2	0.22	2	2	0.21	2	2	0.26
R%	0	0		0	0		0	0		0	0	
AMP												
MIC $_{50}$	4	1		1	1		1	1		1	1	
MIC $_{90}$	4	>32	0.38	3.4	>32	0.23	1	>32	0.42	1	>32	0.26
R%	0	16.9		0	12.9		2.3	11.1		0	19.5	
AUG												
MIC $_{50}$	4/2	1/0.5		1/0.5	1/0.5		1/0.5	1/0.5		1/0.5	1/0.5	
MIC $_{90}$	4/2	16/8	0.64	23/11.5	16/8	0.23	1/0.5	16/8	0.41	1/0.5	16/8	0.39
R%	0	7.4		14.2	5.6		0	0		0	4.1	
AXO												
MIC $_{50}$	<0.25	<0.25		<0.25	<0.25		<0.25	<0.25		<0.25	<0.25	
MIC $_{90}$	0.25	0.25	0.64	0.25	0.25	0.62	0.25	0.25	1.0	0.25	0.25	0.60
R%	0	7.9		0	5.4		1.1	0.5		0	3.0	
FOX												
MIC $_{50}$	4	4		4	4		4	4		4	4	
MIC $_{90}$	4	8	0.64	23.6	8	0.23	4	4	1.0	4	4	0.60
R%	0	8.4		14.2	5.6		1.1	0.5		0	3.0	

Appendix 5. 5 (continued)

Antimicrobial and parameter[a]	Farm						Slaughter					
	Pig			Environment			Carcass			Environment		
	ABF[a] n=5	Conv[b] n=189	*P*	ABF n=14	Conv n=439	*P*	ABF n=86	Conv n=197	*P*	ABF n=63	Conv n=97	*P*
TIO												
MIC $_{50}$	0.5	1	0.64	0.5	1	0.62	1	1	0.41	1	1	0.60
MIC $_{90}$	0.8	2		1	1		1	1		1	1	
R%	0	7.9		0	5.0		0	0.5		0	3.0	
CHL												
MIC $_{50}$	8	8	0.53	8	8	0.53	8	8	0.32	8	8	0.39
MIC $_{90}$	8	8		8	8		8	>32		8	8	
R%	0	5.2		0	4.1		1.1	10.6		0	4.1	
CIP												
MIC $_{50}$	<0.015	<0.015	0.2	<0.015	<0.015	0.2	<0.015	<0.015	0.2	<0.015	<0.015	0.2
MIC $_{90}$	0.015	0.018		0.015	0.015		0.015	0.015		0.0285	0.015	
R%	0	0		0	0		0	0		0	0	
FIS												
MIC $_{50}$	64	64	0.10	64	64	0.03	64	256	0.01	128	64	0.83
MIC $_{90}$	102.4	>256		198.4	>256		64	>256		>256	>256	
R%	0	48.1		0	49.4		1.1	63.4		4.7	16.4	
GEN												
MIC $_{50}$	0.5	<0.25	0.22	0.5	<0.25	0.22	<0.25	0.5	0.41	<0.25	<0.25	0.42
MIC $_{90}$	1	0.5		1	0.5		0.5	0.5		0.5	0.5	
R%	0	0		0	0		0	2.0		0	0	

Appendix 5. 5 (continued)

Antimicrobial and parameter[a]	Farm						Slaughter					
	Pig			Environment			Carcass			Environment		
	ABF[a] n=5	Conv[b] n=189	*P*	ABF n=14	Conv n=439	*P*	ABF n=86	Conv n=197	*P*	ABF n=63	Conv n=97	*P*
KAN												
MIC_{50}	64	<8		8	8		8	8		8	8	
MIC_{90}	>64	8		8	8		8	8		8	8	
R%	60	0	0.01	0	3.8	0.53	0	2.3	0.41	0	0	0.61
NAL												
MIC_{50}	8	4		3	4		2	4		4	4	
MIC_{90}	8	4		4	4		4	4		4	4	
R%	0	3.1	0.87	0	2.5	0.57	0	2.3	0.2	0	0	0.4
STR												
MIC_{50}	32	32		32	32		32	32		32	32	
MIC_{90}	32	32		32	>64		32	>64		32	32	
R%	0	8.9	0.38	0	16.6	0.2	1.1	45.6	0.01	4.7	5.1	0.93
TET												
MIC_{50}	32	32		4	32		4	32		4	4	
MIC_{90}	>32	>32		32	>32		32	>32		32	32	
R%	60	82	0.41	21.4	88.1	0.006	18.6	78.6	0.005	28.5	34	0.27

Appendix 5. 5 (continued)

[a]MIC: minimum inhibitory concentration; Antimicrobials: AMI: Amikacin, AMP: Ampicillin, AUG: Amoxicillin/Clavulanic Acid, AXO: Ceftriaxone, CHL: Chloramphenicol, CIP: Ciprofloxacin, FIS: Sulfisoxazole, FOX: Cefoxitin, GEN: Gentamicin, KAN: Kanamycin, NAL: Nalidixic acid, STR: Streptomycin, SXT: Trimethoprim/sulfamethaxazole, TET: Tetracycline, TIO: Ceftiofur; % R: percent resistance.

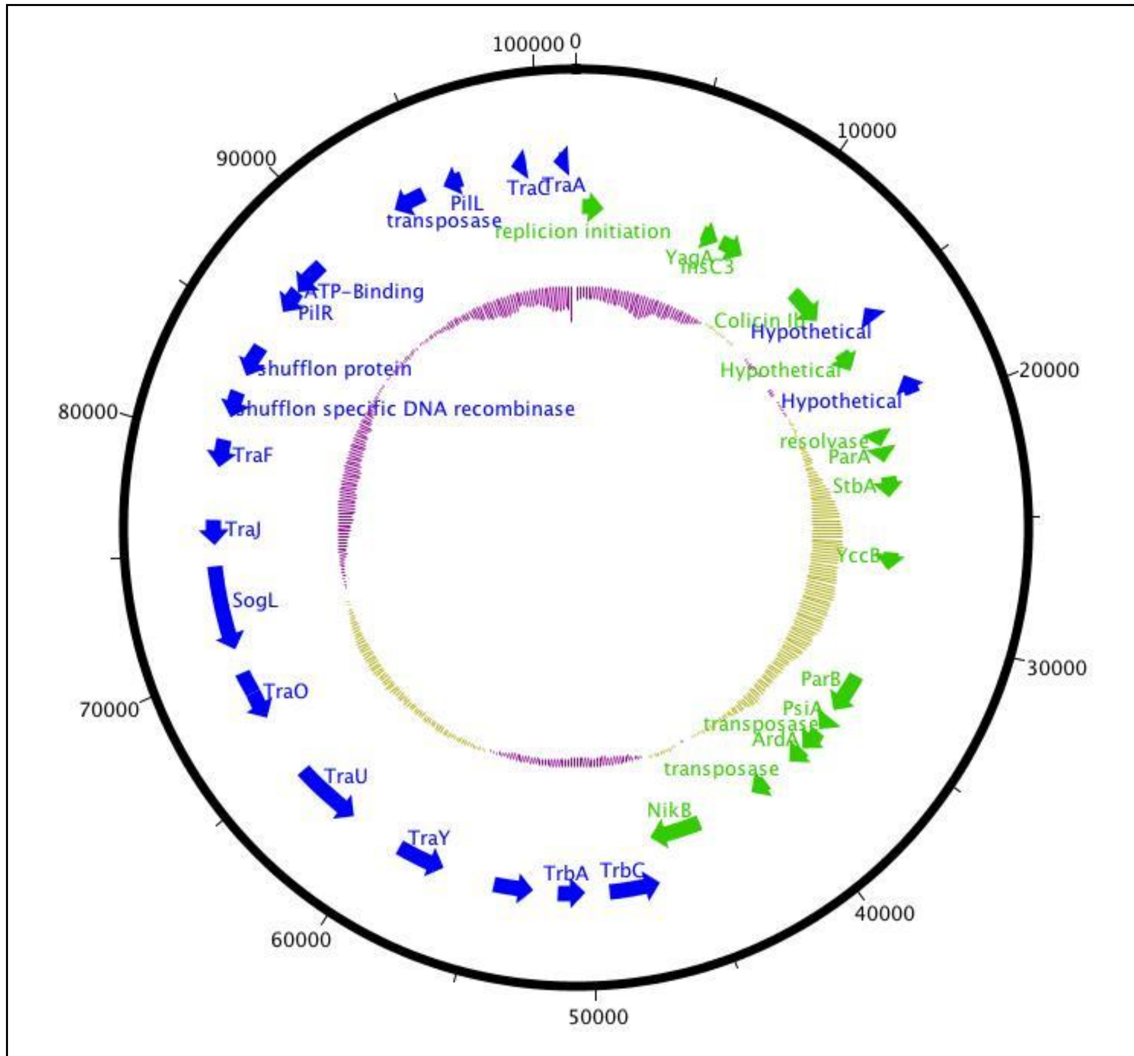
[b]ABF: Antimicrobial free; conv: conventional.

Appendix 5. 6 Geographical distribution of *Salmonella* isolates from humans, pigs and the environment.

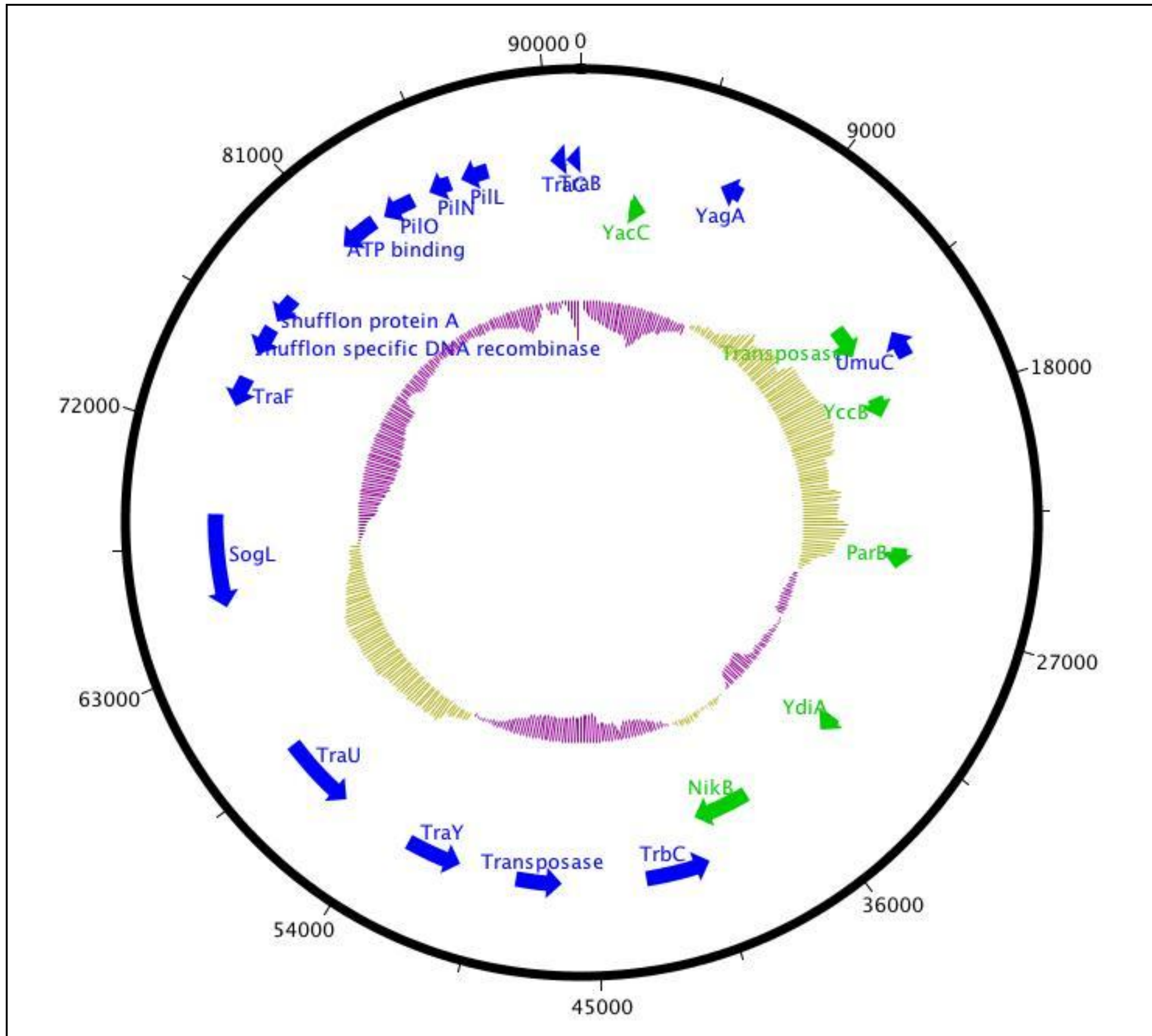
Serotypes	Host and county distribution		
	Humans (n=572)	**Pigs (n=212)**	**Environment (n=274)**
***S*. Typhimurium** (H=290; P=126; E=184)	Alamance (5), Anson (4), Beaufort (1), Bertie (1), Brunswick (3), Cabarrus (9), Cardwell (1), Chatham (3), Cherokee (2), Cleveland (2), Columbus (3), Carven (8), Cumberland (17), Dare (1), Davidson (2), Davie (1), Duplin (1), Durham (14), Edgecombe (3), Forsyth (7), Franklin (5), Gaston (5), Granville (1), Guilford (11), Halifax (1), Harnett (4), Henderson (1), Hyde (1), Iredell (1), Johnston (6) Macon (1), Mecklenburg (46), Mitchell (1), Moore (5), Nash (4), Newhanover (2), Onslow (8), Orange (3), Out of State (6), Pamlico (1), Pender (3), Pitt (1), Randolph (4), Robeson (5), Rockingham (1), Rowan (3), Rutherford (1), Sampson (2), Scotland (1), Stanly (9), Stokes (1), Surry (1), Transylvania (2), Union (2), Unknown (8), Vance (2), Wake (29), Watauga (2), Wayne (2), Wilson (4), Yadkin (2)	Bladen (7), Cumberland (6), Duplin (3), Johnston (20), Sampson (90)	Bladen (3), Columbus (2), Cumberland (8), Duplin (10), Johnston (45), Sampson (116)
***S*. Muenchen** (H=79; P=3; E=0)	Alamance (1), Brunswick (2), Buncombe (1), Burke (1), Cabarrus (9), Cartert (1), Caswell (1), Columbus (2), Carven (2), Cumberland (2), Duplin (2), Durham (1), Forsyth (3), Lincoln (1), Mecklenburg (13), Montgomery (1), Moore (1), New Hanover (8), Onslow (3), out of State (1), Pender (4), Robeson (3), Rutherford (3), Sampson (2), Union (1), Unknwon (2), Wake (5), Wayne (2)	Johnston (3)	N/A

Appendix 5. 6 (continued)

***S*. Typimurium Var C** (H=135; P=2; E=0)	Alamance (5), Ashe (2), Buncombe(1), Burke (2), Cabarrus (6), Cardwell (1), Camden (1), Carteret (1), Catawba (1), Chatham (3), Columbus (3), Carven (7), Cumberland (4), Davidson (1), Durham (3), Edgecombe (2), Forsyth (2), Franklin (1), Gaston (1), Gates (1), Guilford (2), Halifax (1), Harnett (1), Hoke (1), Iredell (2), Johnston (3), (1), Person (1),Pitt (1), Randolph (5), Richmond (3), Robeson (3), Rockingham (5), Rowan (1), Stanly (2), Stokes (1), Transylvania (1), Union (4), Unknown (7), Vance (1), Wake (17), Watauga (2), Wayne (1), Wilson (4)	Sampson (2)	N/A
***S*. Derby** (H=12; P=74; E=32)	Anson (2), Cumberland (1), Carteret (1), Pender (3), Pitt (1), Rutherford (1), Sampson (1), Union (1), Unknown (1)	Johnston (4), Sampson (70)	Johnston (26), Sampson (6)
***S*. Heidelberg** (H=50; P=0; E=17)	Carteret (2), Cherokee (1), Cumberland (2), Durham (1), Edgecombe (2), Forsyth (1), Gaston (1), Guilford (4), Halifax (1), Harnett (1), Henderson (1), Hyde (1), Johnston (1), Mecklenburg (7), Nash (1), New Hanover (1), Out of State (3), Randolph (1), Rockingham (2), Rutherford (1), Stokes (1), Unknwon (1), Vance (3), Wake (3), Wayne (2), Wilson (1)	N/A	Cumberland (1), Johnston (16)
***S*. Schwarzengrund** (H=5; P=0; E=2)	Ashe (1), Cumberland (1), Forsyth (1), Lincoln (1), Pitt (1)	N/A	Duplin (2)
***S*. Rissen** (H=1; P=7; E=39)	Mecklenburg (1)	Cumberland (6), Sampson (1)	Cumberland (33), Sampson (6)



Appendix 5. 7 Circular map of plasmid I1-Iγ (103kb) isolated from *Salmonella* serovar Anatum of pig origin (S732/pS02) containing placement of 35 genes annotated based on BlastX matches



Appendix 5. 8 Circular map of plasmid (91kb) isolated from *Salmonella* serovar Rissen of environment origin (S1004/pS17) containing placement of 35 genes annotated based on BlastX matches