ABSTRACT

PORNSUKAROM, SUCHAWAN. Genomic Characterization of Antimicrobial Resistant *Salmonella* Serotypes across Human, Animal and Environmental Sources Reveals Genome Relatedness and Interserovar Exchange of Resistance Determinants. (Under the direction of Dr. Siddhartha Thakur).

Salmonella is a significant foodborne pathogen. Spread of antimicrobial resistant (AMR) foodborne pathogens due to the antimicrobial misuse in livestock production results in the development of AMR bacterial strains. Land application of animal manure, a practical use in recent sustainable agricultural management, potentially leads to the dissemination of AMR pathogens in the environment and poses a serious public health threat.

To assess the impact of manure application in commercial swine farms on the transmission of AMR *Salmonella* in the environment, we collected environmental samples from farms in North Carolina (*n*=6) and Iowa (*n*=7). The manure and soil samples (before/after manure application) were collected on day 0. Subsequent soil samples were recollected on days 7, 14, 21 from the same plots. A total of 1,300 soil samples and 130 manure samples were included. The overall *Salmonella* prevalence was 13.22%, represented by 10.69% and 38.46% prevalence in soil and manure, respectively. The prevalence in NC (25.45%) was significantly higher than in IA (2.73%) ($P<0.001$). *Salmonella* serotypes detected in NC were not detected in IA. Antimicrobial susceptibility was tested by broth microdilution. A high frequency of isolates (58.73%) were multidrug resistant and the most frequent resistance was detected against streptomycin (88.36%), sulfisoxazole (67.2%), and tetracycline (57.67%). Genotypic characterization by pulsed-field gel electrophoresis (PFGE) revealed clonally related *Salmonella* in manure and soil at multiple time points.

The fourteen isolates of multiple *Salmonella* serotypes including Johannesburg (*n*=2), Ohio (*n*=2), Rissen (*n*=1), Typhimurium var5- (*n* =5), Worthington (*n*=3), and 4,12:i:- (*n*=1),

representing different farms in NC, were selected for characterization of the plasmids carrying AMR determinants. The 14 confirmed transconjugants were tested for antimicrobial susceptibility. The plasmids were isolated by modified alkaline lysis, and PCRs were performed to identify the AMR determinants and the plasmid replicon types. The plasmids were sequenced for further analysis. A class 1 integron with an ANT(2")-Ia-*aad*A2 cassette was detected in the 50-kb IncN plasmids identified in *S.* Worthington isolates. We identified 100-kb and 90-kb IncI1 plasmids in *S.* Johannesburg and *S.* Rissen isolates carrying the *bla*CMY-2 and *tet*(A) genes, respectively. An identical 95-kb IncF plasmid was widely disseminated among the different serotypes and across different farms.

Whole genome sequencing (WGS) was applied to study the association between human *Salmonella* clinical isolates and the environmental/animal reservoirs. *Salmonella* isolates recovered from different sources including human (*n*=44), swine (*n*=32), poultry (*n*=22), and environment (*n*=102) were sequenced. The assembled genomes were used for *in silico* analysis of virulence genes in the VFDB database, and phylogenetically clustered using core genome single nucleotide polymorphism (SNP) and feature frequency profiling. Furthermore, AMR was analyzed by genotypic prediction using five curated AMR databases, and compared to phenotypic AMR using broth microdilution. Both core genome SNP-based and FFP-based phylogenetic trees showed consistent clustering of isolates into the respective serotypes, and suggested clustering of isolates based on the source of isolation. The overall correlation of phenotypic and genotypic AMR was 87.61% and 97.13% for sensitivity and specificity, respectively. AMR and virulence genes clustered with the *Salmonella* serotypes, while there were also associations between the presence of virulence genes present in both animal/environmental isolates and human clinical samples.

In conclusion, the study highlights the potential role of swine manure application in the dissemination and persistence of AMR *Salmonella* in the environment and provided the important evidence of horizontal dissemination of resistance determinants through plasmids of multiple *Salmonella* serotypes across commercial swine farms after land application. Finally, our study supports that the isolates from human had a close relationship with animal and environment isolates which are considered to be the sources for dissemination of AMR and virulence genes between *Salmonella* serotypes.

Genomic Characterization of Antimicrobial Resistant *Salmonella* Serotypes across Human, Animal and Environmental Sources Reveals Genome Relatedness and Interserovar Exchange of Resistance Determinants.

by
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DEDICATION

To My Parents

Noppadol and Suphannee Pornsukarom

for their unconditional love and everything they have done for me.

BIOGRAPHY

Suchawan Pornsukarom was born March 29th, 1984 in Bangkok, Thailand. She attended Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand where she graduated with Doctor of Veterinary Medicine in 2008. She worked as a technician at LAB INTER CO., LTD, Bangkok and INTEQC group, Samut Sakorn, Thailand, 2008-2010. She was granted a full scholarship year 2010 by the Royal Thai Government, Ministry of Science and Technology to study in the graduate program (Master and Doctoral). She pursued Master of Public Health, Veterinary Public Health specialization at the Ohio State University, Columbus, Ohio, from 2011-2013. She joined the Comparative Biomedical Sciences program at North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina in 2014. Her Ph.D. project focused on Genomic characterization of antimicrobial resistant *Salmonella* serotypes across human, animal and environmental sources reveals genome relatedness and interserovar exchange of resistance determinants, under the direction of Dr. Siddhartha Thakur. She will further her career as a lecturer and researcher at Faculty of Veterinary Medicine, Rajamangala University of Technology Tawan-ok, Chonburi, Thailand.

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"Things are seem impossible until they are done."

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. CHAPTER I: Literature Review

1.1. Introduction

The World Health Organization (WHO) estimates that in 2010, 600 million people suffered from foodborne illnesses and 420,000 deaths were reported globally (1). The Centers of Disease Control and Prevention (CDC) reported that, in the U.S.A., there were 902 foodborne disease outbreaks, resulting in 15,202 illnesses, 950 hospitalizations, 15 deaths, and 20 food product recalls in 2015 (2). The diseases are predominantly associated with animal-originated and/or plant-based foods contaminated with pathogenic microorganisms. The enteric zoonotic pathogens which are commonly associated with animal hosts have been identified as causative agents in these food-borne illnesses or outbreaks. Transmission of zoonotic pathogens from animals, plant systems to human occurs by multiple distinct routes, and the application of animal manure into the environment has been suspected to be a contributing factor (3).

Land application of animal manure is an integratl part of the sustainable agricultural management that improves soil fertility and productivity. However, animal feces or runoff carrying enteric zoonotic pathogens have been implicated in some of the foodborne outbreaks in recent years (4, 5). The most common foodborne bacterial pathogens isolated from food animals and their manure include Shiga-toxin producing *Escherichia coli*, *Salmonella*, *Campylobacter*, and *Yersinia* (6, 7). These bacterial pathogens can survive, even multiply in environments after manure application on land, and may cause foodborne illness in human who are directly or indirectly exposed to contaminated manure and food products (8, 9).

The development of antimicrobial resistance (AMR) in foodborne pathogens

associated with food animals has a great impact on a global public health. AMR transmission from livestock farms to agricultural fields and the horizontal gene transfer (HGT) through transmissible plasmids is responsible for the increase of AMR dissemination in foodborne bacterial pathogens (10, 11). Conjugation, transformation, and transduction are the primary mechanisms by which dissemination of AMR genes occurs (12). Human and animals are linked to each other through the environmental reservoirs which have long been implicated as a source of AMR found in human and animals (13, 14). In order to better understand the complex transmission route of AMR foodborne pathogens within farm animals and humans, more studies should be conducted on actual commercial farms.

1.2 Agricultural production and environmental impact

Intensification of agricultural production over the world has been driven by a large use of non-renewable resources that often affect environmental sustainability at all levels including field, livestock farm, landscape scales (15). Particularly in both industrialized and developing countries, agriculture has become highly specialized to improve the productivity to fulfill the population growth, political need, and economic demand (16, 17). The agricultural intensification and specialization has a negative influence on the environment and have been not fully accepted by the society (15). Recently, sustainable agricultural systems have become more popular due to productivity and environmental favor. The crop-livestock integrated systems, combining livestock production and harvesting at farm level, are considered to be a suitable way to achieve sustainable by improving nutrient cycling and decreasing environmental fluxes with potential socio-economic (16, 18).

1.2.1 Animal manure application

Livestock production in the U.S. generates more than a billion tons of manure Annually, including from cattle (83%), swine (10%) and poultry (7%) productions (19). In order to deal with a number of livestock manure according to sustainable management, farmers stack it in piles or store it in lagoons, and apply it on agricultural field by spreading or injecting into the soil for nutrient recycling. Injection method creates a furrow into the ground and fills manure within soil. This method is efficient at ammonium utilization, bypassing infiltration, less contact to atmosphere, and good to environment, however, high energy-demand and specific soil requirement are drawbacks (20, 21). Liquid manure stored in lagoons is generally applied to the land through the use of irrigation sprinklers. This is a cheapest method which dilutes manure into a number of small droplets or aerosols and sprays to the field leading to increase in potential threat to the environment and human health problems (21). Swine manure is usually stored and treated in anaerobic lagoons or manure pits before land application as crops fertilizer (22). The type of waste management system used on each farm depends on type of housing, manure storage, land application, and geographic location. The pit systems are commonly used in the north-central region where the temperature is too cold to maintain a manure lagoon (23). The purposes of an anaerobic lagoon system is to decompose animal manure and convert manure to liquid for transportation and application (23, 24). The significant environmental concerns including odors, overflow, potential leakage, and over application of lagoon effluent are considered in anaerobic lagoon system (21, 23). However, the animal manure application is economical

and beneficial for integrated farm management and we have to pay attention to the environmental concerns and pathogen spreading after manure application.

1.2.2 Spread of bacterial pathogen by manure application

Land application of livestock manure in commercial and organic farms is an integral part of sustainable soil management. However, it can lead to the dissemination of pathogenic bacteria in the environment causing a public health problem. Several studies designed to determine the role of swine manure in pathogen transmission to the environment were either conducted on a few commercial farms (25, 26) or on experimental research stations (27-29). A number of studies have reported the presence of multiple bacterial pathogens including *Escherichia coli*, *Campylobacter*, *Listeria*, and *Salmonella* in effluent lagoons and the persistence of these pathogens on land after manure application (9, 30, 31). Garcia et al. observed *Salmonella* survival at 5°C-25°C in the topsoil inoculated dairy cow manure (32). After 28 days of manure application, *Salmonella* were recovered in applied soil and the concentrations were also higher in loamy soils compared to sandy soils (33).

Antimicrobial resistant bacterial pathogen has been among the major public health concerns worldwide. There are numerous studies reporting the prevalence of AMR foodborne pathogens including *Salmonella*, *Escherichia coli* and *Campylobacter* among swine reared in the commercial production systems (26, 31, 34). Moreover, the lagoon, manure slurry, as well as amended soil following land application were observed to be the source of pathogens and AMR genes (35-38). Our study reported the dissemination and persistence of resistant *Salmonella* in the environment of commercial swine farms after manure application on land (9).

1.3 *Salmonella*

Since 1950's, salmonellosis has been recognized as a major foodborne disease among pathogenic bacteria causing a number of incidences and outbreaks (39, 40). In the U.S., it was estimated that more than 100,000 illnesses each year and more than 40 deaths occur as a result of salmonellosis (39). This causative agent was reported as the second most often in European region with 91,408 clinical cases of the foodborne outbreaks recorded by European Centre for Disease Prevention and Control (40).

Salmonella is gram negative, motile, medium rod-shaped (1×4 μm), non-spore forming, and facultative anaerobic bacteria belonging to family *Enterobacteriaceae*. They are mesophile, with 5-46°C temperature range for growth, but they grow optimally at 35-37°C (41). Even though *Salmonella* does not ferment lactose, sucrose, or salicin, they generally produce gas by using monosaccharide like glucose as a source of energy (42). When using citrate as carbon source, they produce hydrogen sulfide. They decarboxylate lysine and ornithine, cannot produce indole, and do not test positive for catalase, oxidase and urease tests (41, 43). Pasteurization and acid (pH lower than 4.5) can destroy *Salmonella*. However, *Salmonella* can survive under freezing and dried conditions for a long period of time and multiply in various kinds of food, except at water activity (A_w) less than 0.94 (41). According to WHO Collaborating Centre for Reference and Research on *Salmonella*, there are more than 2,600 serovars of *Salmonella* (44, 45). This genus contains two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is divided into six subspecies: I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S.enterica* subsp. *indica*

(44, 46). The species called *S. subterranean* does not belong in the genus *Salmonella* (44, 47). The characterization of *Salmonella* serovar is based on the White-Kauffmann-Le Minor scheme depending on O and H antigens (45).

1.3.1 *Salmonella* in humans, animals, and environment

Most of the 2,600 serovars of *Salmonella* inhabit in human, domestic and wild animals' gastrointestinal tracts and can be found in environment. Majority of serovars belong to *Salmonella enterica* subsp. *enterica* which potentially causes salmonellosis in humans and animals (44). Foodborne gastrointestinal illness caused by *Salmonella* typically results in abdominal cramps, profuse diarrhea, nausea, headache, vomiting, acute onset of fever and prostration. The symptoms appear within 8-42 hours, most commonly within 24-36 hours after ingestion and usually last for 2-3 days (41). The level of severity depends on the dose of ingestion and individual immunity (48). It can be fatal in sick people, infants, and elderly (48). The most frequent serovars causing human illness reported by CDC are Enteritidis (16.5%), Typhimurium (13.4%), Newport (11.4%), and Javiana (6.4%) (49, 50). Additionally, multiple *Salmonella* serovars, including Agona, Anatum, Derby, Heidelberg, Infantis, Kentucky, Muenchen, Newport, Schwarzengrund, and Typhimurium are commonly detected in food animals, food products, agricultural environment and are responsible for AMR *Salmonella* infection in human (49, 51, 52). *Salmonella* are ubiquitous and survive for a long time in natural environment with the favorable conditions (53). Previous study reported that *Salmonella* survived in fresh water around 2 months and several years in soil (54). Both clinical and non-clinical *Salmonella*-infected human, as well as animals can excrete *Salmonella* through household runoff, livestock waste, and agriculture dissemination

(55). *Salmonella* infects people by fecal-oral route. However, most of human salmonellosis cases are associated with consumption of contaminated animal-origin products (56). *Salmonella* serovars of food animal origin are introduced to a food chain and spread to population through food production.

1.3.2 *Salmonella* in food

Products of animal origin including beef, chicken, turkey, pork, eggs, and milk are associated with large number of human salmonellosis (57, 58). In Europe and North America, contaminations in egg and poultry have been considered as the main source of infection (57, 59). In Denmark, people were infected *Salmonella* by table egg (47%), followed by domestically produced pork (9%) and imported poultry (7%) (60). In Thailand, *Salmonella* has been isolated from many foods of animal-origin including milk, chicken and pork (61, 62). Several *Salmonella* serovars were observed in seafood and caused plenty of salmonellosis outbreaks such as finfish harvested from polluted water (63, 64). *Salmonella* intentionally or accidentally contaminated food products by fecal-oral route. Consequently, contaminated food products were eaten raw, undercooked or cross contamination after adequate heat treatment (57). In addition, *Salmonella* was also isolated from many food types of plant-origin including cantaloupe, tomato, and nut. The previous outbreaks in vegetables and fruits in the U.S. were *Salmonella* Saintpaul among 18 states in April, 2013 which were associated with imported cucumbers from Mexico caused 28 illnesses and 26% were hospitalized (65). *Salmonella* Typhimurium and *Salmonella* Newport infection linked to cantaloupes caused 261 cases, 94 hospitalizations, and 3 deaths among 24 states in August, 2012 (66). *Salmonella* has the ability to attach and internalize into fresh produce and cause

infection (65). *Salmonella* contamination occurred due to environmental factors including manure application, using sewage as fertilizer or washing products with unclean water (3, 67). Not only contaminated chicken and egg play the most important role in *Salmonella* illness worldwide, but also pork and pork products also are determined as a major vehicle of human salmonellosis (68). It is estimated that 15-20% of salmonellosis cases in human are the result of pork consumption (68). *Salmonella* contamination can occur in any stage of pork production line, including at the farm and slaughter process that can easily spread to consumers through the food chain (69, 70).

1.3.3 *Salmonella* in swine production

The *Salmonella* serovars commonly recognized in swine production are the major cause of public health problems. Because of the overlap of the serovars between pig and human infections, there is strong potential tha the pathogen can spread across species through food production and agricultural farms (52). According to the CDC surveillance (2011), the most frequent *Salmonella* serotypes isolated from pork products causing clinical illness were Typhimurium, Derby, Agona, Infantis, and Heidelberg (50), whereas the CDC surveillance (2005), it was pointed out that Typhimurium and Cholereasuis tended to be more pathogenic than other serovars (71, 72). *Salmonella* Typhimurium is still a major problem year after year, while prevalence of *S.* Cholereasuis is decreasing.

Salmonella prevalence is common in commercial swine production systems in many parts of the world (70). The prevalence varies by different factors including farm surveyed, rearing process, density, and herd size. In the U.S., a report found that 0.2-20% of pigs and fecal samples were positive for *Salmonella* (73, 74). The stage of production has an impact

on the percentage of *Salmonella* prevalence. The prevalence in gilt farms was 3.4%, while the prevalence in breeder farms was 18 to 22% (75). *Salmonella* prevalence increased significantly from late nursery to slaughter (76). Previous study found that the pig reared in the conventional farm showed the higher prevalence of *Salmonella* in term of fecal samples and carcass samples than those from antimicrobial free farms (73). Contrast to the surveillance previously done by Davies et al., in developing countries, the *Salmonella* prevalence was as common as the developed countries, regardless of whether samples originated from intensive swine industries or not (77). The fecal samples collected on commercial swine farms were 0-42% positive, while the samples collected in the slaughterhouse were 0-77% positive (73, 78). Consequently, the herd showing high positive percentage of fecal sample tended to have a high *Salmonella* contamination rate on the carcass (78). The prevalence of *Salmonella* in retail pork products was reported from 6.32% to 39.6% (79-81). The highest percentages found in ground pork (16%) because this kind of product was combined of multiple pigs and several parts of swine including visceral organs (82). The lowest positive percentages were in pork chops (1-33%) (82). *Salmonella* on fattening pigs skin or in intestinal content and microflora found on slaughter equipment have been shown to contaminate the belly opening and carcass splitters (83).

1.3.4 Antimicrobial resistance (AMR) in *Salmonella*

Antimicrobial agents are widely used in human and veterinary medicine. There are three main purposes of antimicrobial use in food animal production: disease therapeutics, infection prophylaxis, and growth promotion (84). There is the increased overall use of antimicrobial in food animal production. Thus, the problem of antimicrobial resistant

Salmonella has doubled generally over the last few decades (85). In the United States, more than 80% of *Salmonella* isolated from meat products including chicken, pork, and beef showed resistance to at least one antimicrobial and approximately 50% showed resistance to at least three antimicrobials (86, 87). In Southeast Asia, 35%-40% of *S. enterica* isolates collected from swine source and 42% from healthy human source were multiple antimicrobials resistance (MDR) (88, 89). Moreover, *S. enterica* also commonly found in chickens, pigs, cows, farm workers, and diarrheal children (88, 89). In Vietnam, 78% of *Salmonella* isolates from retail meat samples were resistance to at least one class of antimicrobials and the highest frequency of resistance was detected against tetracycline, sulfonamides, streptomycin, ampicillin and chloramphenicol (90, 91). Resistance to tetracycline was the greatest observed in isolates from fresh pork sausages in Brazil (92). Resistance to tetracycline, streptomycin, and sulfamethaxazole were presented widespread in the isolates from both healthy and diarrheal pigs, meat products and patients with diarrhea (73, 87, 89, 93). Though human salmonellosis is generally a self-limiting disease and treatment would be needed only in severe cases, the increase of multidrug resistance (MDR) *Salmonella* including fluoroquinolones and extended-spectrum β-lactams (ESBL) makes infections more complicated (94). Additionally, MDR *Salmonella* infections lead to treatment failure, prolonged hospitalization and increase of economic loss in public health. As discussed previously, AMR *Salmonella* and their determinants could enter the environment following the land application of animal manure. Studies have reported the presence of AMR *Salmonella*, AMR genes, antimicrobial residues in lagoons and on lands that have been exposed to the swine manure (95, 96). The aqueous concentrations of

chlortetracyline and tylosin resulted in runoff from swine manure and significantly *erm* genes were found while no clear trend of AMR gene was observed on topsoil following manure application (35).

Salmonella becomes antimicrobial resistant due to various mechanisms including enzymatic antimicrobial inactivation, modification/protection of target sites, limiting antimicrobial access to microbial cell, and active efflux (97, 98). Among those mechanisms, the presence of integrons and overexpression of active efflux is known as the main causes of MDR in *Salmonella* (99). Integrons are mobile genetic elements located on *Salmonella* chromosome or conjugative plasmid. In gram negative bacteria, a class 1 integron is the most common type carrying various resistance gene cassettes and responsible for horizontal transfer (99, 100). The presence of *Salmonella* genomic island1 (SGI1), which is chromosomally located, typically limits to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline resistances. Because of SGI1, *Salmonella* is able to maintain the resistance determinants without selective pressure (97). Unlike integrons, active efflux pumps are not structurally related, thus the cross-resistance to MDR can occur. The efflux pumps decrease the gradients of antimicrobial to insufficient level which not harmful to bacterial cells and support the mechanism of MDR (97). The AMR genes occur via mutation in DNA and horizontal gene transfers by transformation, transduction and conjugation (100, 101). The clonal spread and horizontal gene transfer are considered as major route of AMR distribution among *Salmonella* (101, 102).

1.4 Plasmid replicons and horizontal gene transfer

The dissemination of AMR in gram negative bacteria has been largely attributed to

horizontal transfer of plasmid-located AMR gene (103). Plasmids are extra chromosomal circular elements of DNA capable of autonomous replication and are transferable between different bacterial species. Plasmids appear to increase bacterial genetic diversity containing backbone genes, which are required for plasmid survival, transfer, and horizontal exchange by conjugation or mobilization (104). Plasmids also contain accessory genes encoding for AMR or virulence genes which are beneficial to the bacteria (105). Since 2005, plasmids were identified and characterized to incompatibility (Inc) groups using PCR-based replicon typing (PBRT) scheme (106). The incompatibility is the inability of two related plasmids to coexist in the same host cell which based on stability during conjugation period. The PBRT classified plasmids of *Enterobactericeae* into major plasmid families including FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA (106).

Horizontal gene transfer plays an important role in the rapid spread of AMR determinants to susceptible bacterial population. Plasmid conferring resistance has been identified as the principle of clinically severe AMR, including β-lactams, cephalosporins, and quinolones (107). *Salmonella* isolates recovered from human, animals, environment and food contained plasmids carrying at least one β-lactamase-encoding gene (*bla*TEM, *bla*PSE-1, *bla*SHV, or *bla*CTX-M and the plasmid-mediated quinolone resistance (PMQR; qnrA, qnrB, qnrS) genes (108, 109). A study conducted by Han et al. reported that *Salmonella* Heidelberg isolated from human, dairy, pig, and turkey harbored plasmids containing AMR and virulence genes that were able to mediate the horizontal transfer of plasmid within and among various bacterial isolates (110). AMR determinants dissemination present in plasmids of multiple *Salmonella* serotypes in the environment after manure application.

1.5 Whole genome sequencing (WGS)

A number of AMR *Salmonella* studies have used the classical molecular typing methods such as pulse-field gel electrophoresis (PFGE), multilocus sequence-based typing (MLST), and multilocus variable-number tandem repeat analysis (MLVA) to assess the relatedness and the subsequent transmission of AMR *Salmonella* in human, animals, and environment (111-113). However, there are limitations of these methods in terms of insufficient discriminatory power to separate closely related *Salmonella* isolates in outbreak investigations and to differentiate between the intra-serotype isolates from different hosts (112-114). In addition, WGS offers the highest practical resolution characterization including AMR tested phenotypically. Thus, the WGS was widely introduced to AMR *Salmonella* studies.

WGS allows researchers to routinely monitor *Salmonella*, detect its outbreak, analyze antimicrobial resistance, and predict the resistance phenotypes. The single nucleotide polymorphism (SNP)-based phylogenetic methods that relies on WGS can be effectively utilized for determining the phylogenetic clonal isolates of *Salmonella enterica* serotypes including Typhimurium (115), Entritidis (114), Heidelberg (116), Manhattan (113), and Montevideo (117). The SNP analysis approach can compete with the classical methods that are commonly used in *Salmonella* detection and outbreak investigation, such as PFGE and MLVA. PFGE has been a CDC gold standard method for foodborne bacteria characterization. Because of its limitation of electrophoresis mobility, PFGE cannot separate the closely related strains (118). MLVE are useful in epidemiological surveillance but the lack of serotype specific protocols is its drawback (119). A WGS study in *S*. Typhimurium

isolates for outbreak investigation in Denmark reported that SNP tree and nucleotide difference tree clustered 100% of *S.* Typhimurium, while pan-genome tree and k-mer tree clustered 65% and 88%, respectively. SNP analysis and nucleotide difference approach are the superior methods for epidemiological typing compared to classical typing method. However, the epidemiological data is still necessary in addition to WGS data because there is some information that cannot be revealed by gene (115). Furthermore, WGS technology can be applied to identify known antimicrobial resistance determinants among strains of nontyphoidal *Salmonella* and predict the susceptibility phenotypes. McDermott et al. claimed that WGS analysis is as reliable as Minimum Inhibitory Concentrations (MICs) using standardized broth microdilution (120). They were able to identify resistance genes and mutations in structural resistance loci via assembled sequences of various *Salmonella* serotypes selected from human clinical cases and retailed meat. The correlation between resistance genotypes and phenotypes was 99%, almost reached 100% in most classes of antimicrobial except the aminoglycosides and β-lactams groups (120). It indicated that k-mers was applied to map the WGS data against the database of resistance genes and revealed that KmerResistance achieved significantly better than other methods. k-mer was a useful tool to identify the resistance genes as well as the species, when dealing with contaminated or losing data (121). WGS was also used in the study of plasmid and chromosomal analysis including the study of β-lactamase-resistant *S.* Typhimurium and *bla*CTX genes (109). Implementation of a validated pipeline for WGS and analysis provided reliable results which are predictable and stable. Thus, the WGS become routine and widespread for multiple kinds of research.

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2. CHAPTER II: Assessing the impact of manure application in commercial swine farms on the transmission of antimicrobial resistant *Salmonella* in the environment.

Presented here is the manuscript titled "Assessing the impact of manure application in commercial swine farms on the transmission of antimicrobial resistant *Salmonella* in the environment", published year 2016 in the journal of PloS one, vol. 11(10), pgs. e0164621. Additional data are presented in the Appendix.

2.1 Abstract

Land application of swine manure in commercial hog farms is an integral part of their waste management system which recycles the nutrients back to the soil. However, manure application can lead to the dissemination of bacterial pathogens in the environment and pose a serious public health threat. The aim of this study was to determine the dissemination of antimicrobial resistant *Salmonella* in the environment due to manure application in commercial swine farms in North Carolina (n = 6) and Iowa (n = 7), two leading pork producing states in the US. We collected manure and soil samples twice on day 0 (before and after manure application) from four distinct plots of lands (5 soil samples/plot) located at 20 feet away from each other in the field. Subsequent soil samples were collected again on days 7, 14, 21 from the same plots. A total of 1,300 soil samples (NC = 600; IA = 700) and 130 manure samples (NC = 60; IA = 70) were collected and analyzed in this study. The overall *Salmonella* prevalence was 13.22% (189/1,430), represented by 10.69% and 38.46% prevalence in soil and manure, respectively. The prevalence in NC (25.45%) was significantly higher than in IA (2.73%) (P<0.001) and a consistent decrease in *Salmonella* prevalence was detected from Day 0-Day 21 in all the farms that tested positive. *Salmonella*

serotypes detected in NC were not detected in IA, thereby highlighting serotype association based on manure storage and soil application method used in the two regions. Antimicrobial susceptibility testing was done by the broth microdilution method to a panel of 15 antimicrobial drugs. A high frequency of isolates (58.73%) were multidrug resistant (resistance to three or more class of antimicrobials) and the most frequent resistance was detected against streptomycin (88.36%), sulfisoxazole (67.2%), and tetracycline (57.67%). Genotypic characterization by pulse field gel electrophoresis revealed clonally related *Salmonella* in both manure and soil at multiple time points in the positive farms. Our study highlights the potential role of swine manure application in the dissemination and persistence of antimicrobial resistant *Salmonella* in the environment.

2.2 Introduction

Every year in the US more than a billion tons of manure is generated by livestock, primarily cattle (83%) followed by swine (10%) and poultry (7%) operations (1, 2). To deal with a large amount of manure, producers stack it in piles or store it in lagoons, and apply it on agricultural land to recycle the nutrients. Before application, the manure is often treated with thermophilic composting to inactivate potential pathogens that may be present (3, 4). However, there are concerns related to the dissemination of pathogenic bacteria from manure-amended soil which can contaminate water, food animals, and crops. Previous studies have reported the dissemination and persistence of infectious pathogens, antimicrobial residues, and antimicrobial resistance genes on agricultural field following manure application that subsequently enter the human food chain to become a public health hazard (5–11). Several studies have reviewed the persistence of *Salmonella* in inoculated soil

under various laboratory conditions over extended periods of time (12–14). A study from Sub-Saharan Africa recovered *S.* Typhimurium six weeks after application of low-density inoculated manure and 14 weeks after application with high-density *Salmonella*-inoculated manure in a tropical climate (15). *Salmonella* Typhimurium has been shown to persist in soil 180 days after application of cattle slurry in Sweden (16).

Manure generated in swine operations is usually collected, stored and treated in anaerobic lagoons or manure pits before being applied to the land as crops fertilizer (17, 18). The type of waste manage system used on each farm depends on multiple factors including the type of animal housing, manure handling during storage and land application, and geographic location. The pit systems are commonly used in the north-central region where the manure can be recycled back to cropland and the temperature is too cold for maintaining a lagoon (19). The conventional approach is the pit-storage system which is located under the building and allows slurry to be stored for 120–180 days before being applied to the field. The pit-recharge system was developed to improve air quality inside livestock building (20, 21). The system, located under the ground, keeps most manure solids in suspension thereby making them easier to remove when the pit is drained. The anaerobic lagoon system was designed for anaerobic bacteria to decompose animal manure and convert manure to liquid that is easier for transportation and application (19, 22, 23). The important environmental concerns with anaerobic lagoons are odors, overflow, potential leakage, and over application of lagoon effluent (19). The method used for manure application depends on the kind, volume and consistency of the manure, the hauling distance, costs, and existing equipment (24). Liquid manure stored in lagoons is usually applied to the land through the use of

irrigation sprinklers. This cheap method dilutes and forms manure into several small droplets or aerosols and has the potential of increased pathogen spreading, odor problems, and environmental concerns (25).

Studies aimed to determine the role of swine manure in the dissemination of *Salmonella* to the environment have either been conducted on a few commercial farms (26, 27) or on experimental research stations (9, 28, 29). The other important concern relates to the potential dissemination of antimicrobial resistant (AMR) pathogens when swine manure is spread in the environment (30, 31). It is evident that there is a dearth of information on the potential movement of *Salmonella* from swine manure to other environmental niches in commercial swine farms. It is important to highlight that most of the studies estimating the dissemination of pathogens in the environment from animal manure are conducted on experimental research stations with spiked manure samples. To address this knowledge gap, we conducted a study in two leading pork producing states (North Carolina and Iowa) in the US to determine whether spreading manure in the environment leads to the dissemination of AMR *Salmonella*. We also compared the waste management system in the two states and its impact on *Salmonella* prevalence, serotype distribution, AMR-patterns, and pulsed-field gel electrophoresis (PFGE) profiles.

2.3 Materials and methods

2.3.1 Farm distribution and waste management system

The sampling was conducted on a total of 13 commercial swine farms, including six sites in North Carolina (NC) and seven sites in Iowa (IA). Access to swine farms was approved by either the swine veterinarian or the farm owner. No samples were collected from

vertebrate animals and the field studies did not involve an endangered or protected species. Protocols were discussed with the concerned authorities before proceeding onto the farm premises for sample collection and processing. In the seven IA farms that were sampled, the waste management system involved the use of a deep pit slurry system to store and treat swine manure. This is the preferred method of waste management system in swine farms in IA. Farms in Iowa store undiluted manure in pits and transfer the slurry to the fields by injection method. Soil injectors place liquid slurry into the soil at approximately 5–10 cm depth and cover by soil after the application. In NC, the farms sampled used an anaerobic lagoon system which is widely used in the state. Farms in North Carolina have wells on their property and use a flush system for dilution and manure removal from the housing to the anaerobic lagoons where they are stored in aerated ponds. Aerosolized lagoon waste is reduced into smaller particulate droplets and sprayed to the agricultural field using an irrigation sprinkler. The typical rate of manure application ranges from 4.2–4.7 liters/m^2 for the injection method to 4.2–6.0 liters/m^2 for the sprinkler approach (32). In general, four slurry application methods are available, including the conventional method of broadcast spreading (splash plate), surface banding with trailing-hose (band spreading), trailing shoes, and injection (25, 33). Injection method creates a furrow into the ground and fills manure within soil. This method is efficient in ammonium utilization bypassing infiltration, less air exposure of slurry, and environment-friendly, however, has a high energy-demand and specific soil requirements (25, 33).

2.3.2 Sample collection

In both the states, we visited each farm multiple times: day 0 (before and after manure application), day 7, day 14, and day 21 to study the potential dissemination and persistence of *Salmonella* in the soil following manure deposition. The soil samples were collected following the approach described previously with a few modifications (27). A total of 1,430 samples were collected in the study, including 1,300 soil (NC: 600; IA: 700) and 130 manure samples (NC: 60; IA: 70). Manure samples (n = 10; 25 ml) were collected from the top 30 cm of lagoons or pits using 120-ml sterile containers during the first visit on each farm. Soil samples were collected twice on day 0 (before and after manure application) from four different plots within 0.4 hectare (4,000m^2) size of land (80 X 50 m). The farms in this study applied manure between 8–11 am in the morning. Before sample collection, 4 plots (1 m^2 each) were identified at 20 feet apart from each other in a straight line and directly in line of the manure applicator. The plots were marked by flags for identification during subsequent sampling periods. We collected five soil samples (25 cm deep) weighing 100 gm each from every plot including the four corners and the middle of the plot. After 1–2 hours of manure application, soil samples were collected again from the same place in the plots (n = 20) on day 0. Overall, we collected a total 40 soil samples on day 0. This was followed by sequential visits on day 7, 14, and 21 to collect soil samples (n = 20) from the same spots. Samples originating in IA were shipped overnight at 4°C to NC and processed immediately in the laboratory.

2.3.3 *Salmonella* isolation and confirmation

All the 1,430 samples were processed in NC for *Salmonella* isolation using standard methods described previously (34–36). Briefly, 90 ml of buffered peptone water (BPW) (Difco, Becton-Dickinson, USA) was added into a Whirl-Pak bag containing 10 g of soil or 10 ml of manure sample and mixed thoroughly to be incubated at 37°C for 24 h. After pre-enrichment, a total of 100 µl of BPW suspension was transferred into 9.9 ml of Rappaport-Vassiliadis (RV) enrichment broth (Difco, Becton-Dickinson, USA) and incubated at 42°C for 24 h. A 10-µl loopful of enriched RV suspension was plated onto xylose lactose tergitol (XLT4) agar (Criterion, Hardy Diagnostics, USA) and incubated at 37°C overnight. A single black-colored colony from XLT4 plate was selected and inoculated by streaking and stabbing into triple sugar iron (TSI) and lysine iron agar (LIA) slants (Difco, Becton-Dickinson,USA) for biochemical testing. The presumptive *Salmonella* isolates that tested positive on TSI and LIA biochemical testing were confirmed by amplification of a targeted *Salmonella*-specific invasive (*inv*A) gene by PCR (37). The isolates confirmed as *Salmonella* were labeled and stored in Brucella broth (Difco, Becton-Dickinson, USA) at -80°C until further characterization.

2.3.4 *Salmonella* serotyping

The Kauffman-White scheme was applied for *Salmonella* serotyping. All *Salmonella* isolates (n = 189) were cultured overnight at 37°C on Luria-Bertani (LB) agar (Criterion,Hardy Diagnostics, USA) and sent to the National Veterinary Services Laboratories (NVSL) at Ames, Iowa for serotyping.

2.3.5 Antimicrobial susceptibility testing

The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and antimicrobial resistance (AMR) profile of all confirmed *Salmonella* isolates recovered from soil and manure. This assay was carried out using Sensititre® gram-negative CMV3AGNF plate (Trek Diagnostic Systems, Cleveland, OH, USA). The panel of 15 antimicrobials, abbreviation and respective concentration ranges, included are: amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 μg/ml), ampicilin (AMP; 1–32 μg/ml), azithromycin (AZI; 0.12–16 μg/ml), cefoxitin (FOX; 0.5–32 μg/ml), ceftiofur (XNL; 0.12–8 μg/ml), ceftriaxone (AXO; 0.25–64 μg/ml), chloramphenicol (CHL; 2–32 μg/ml), ciprofloxacin (CIP; 0.015–4 μg/ml), gentamicin (GEN; 0.25–16 μg/ml), kanamycin (KAN; 8–64 μg/ml), nalidixic acid (NAL; 0.5–32 μg/ml), streptomycin (STR; 32–64 μg/ml), sulfisoxazole (FIS; 16–256 μg/ml), trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 μg/ml), and tetracycline (TET; 4–32 μg/ml). The MICs were determined and breakpoints were interpreted based on the Clinical and Laboratory Standards Institute standards (CLSI) for broth microdilution (38) and National Antimicrobial Resistance Monitoring System (NARMS) (39). *E. coli* ATCC 25922 was used as reference strain to measure sensitivity. The isolates interpreted as intermediate level were categorized into susceptible to avoid overestimation of resistance. The isolates with resistance to three or more classes of antimicrobials were classified as multidrug resistance (MDR).

2.3.6 Pulse field gel electrophoresis (PFGE) analysis

Salmonella isolates from soil (n = 139) and manure (n = 50) recovered from different commercial swine farms in NC and IA were genotyped using PFGE following the PulseNet

protocol from the Centers for Disease Control and Prevention (CDC) (40). In brief, *Salmonella* isolates were grown on LB agar plates at 37°C for 14–18 h. Cell suspension buffer (CSB; 100 mM Tris and 100 mM EDTA, pH 8.0) was used to suspend and adjust the bacterial concentration to an optical density (OD) of 0.48–0.52 using a Dade MicroScan Turbidity meter. TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), cell lysis buffer (CLB; 50 mM Tris, 50 mM EDTA: pH8 and 1% sarcosyl) and proteinase K (20 mg/ml) were used to prepare agarose embedded cells. After the bacterial cells were lysed, intact genomic DNA was digested with 50 U of *Xba*I restriction enzyme (New England Biolabs, Ipswich, MA, USA) at 37°C for 2 h. The PulseNet universal strain *Salmonella enterica* serovar Braenderup H9812 was used as a molecular standard marker. The DNA fragments were separated by CHEF-DR®III Pulsed-Field Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) at 14°C for 18 h. BioNumerics software version 6.1 (Applied Maths, Kortrijk, Belgium) was used to analyzed the PFGE images. The clonal relatedness was determined using the Dice coefficient similarity index and unweighted pair group average (UPGMA) cluster analysis with 2.0% optimization and 2.0% tolerance banding pattern. PFGE fingerprint patterns with a similarity index >90% were clustered within the same genotypic group.

2.3.7 Statistical analysis

Pearson's Chi-square analysis was performed to test difference in *Salmonella* prevalence between sample types (manure and soil), manure storage system (lagoon or pit), and state of origin (NC and IA). A value of $P < 0.05$ was considered statistically significant finding. Strength of association between serotype and AMR pattern was determined using the

odds ratio (OR) with a 95% confidence interval. All data analysis was carried out using R version 3.1.2 (R foundation for statistical computing, Vienna, Austria).

2.4 Results

2.4.1 *Salmonella* prevalence in swine farms environment in NC and IA

A significantly higher prevalence of *Salmonella* was detected in NC (168/660, 25.45%) than IA (21/770, 2.73%) for a total of 189 *Salmonella* isolates in the study ($P<0.0001$). We isolated *Salmonella* from all the six farms tested in NC while only a single farm in IA (IAF 6) tested positive (Fig 2.1; Appendices 1-2). *Salmonella* prevalence in manure (50/130; 38.46%) samples was significantly higher than in soil (139/1,300; 10.69%) ($P < 0.0001$) (Appendix 2). Of the 60 manure samples from NC, 40 (66.67%) were positive for *Salmonella*, while only 10 out of 70 manure samples (14.29%) from IA were positive ($P < 0.0001$). A total of 128 (21.33%) out of 600 soil samples from NC and 11 (1.57%) out of 700 IA soil samples tested positive for *Salmonella*, respectively ($P < 0.0001$).

The prevalence of *Salmonella* in farm environment at different time points following land application were highest on day 0, especially from the manure collected directly from the lagoon/pit and the soil samples collected immediately after manure application. *Salmonella* prevalence tended to decrease in subsequent weeks, except in NCF 3 where the prevalence increased on future samplings done on days 7, 14 and 21 (Fig 2.1). Rarely *Salmonella* was detected on land before manure application, except for a single soil sample collected from NCF4. We detected variation in serotype distribution between farms. For example, in NCF 1, *Salmonella* serotypes Altona, Mbandaka, Muenster, Uganda, and Worthington were recovered in manure lagoon as well as and manure enriched soil on the

first visit but none of these serotypes were recovered from soil before manure application. The five serotypes persisted in the soil samples until day 7. After two weeks, we detected only serotypes *S.* Altona and *S.* Muenster in the soil samples from NCF 1 and finally only *S.* Altona was recovered on the last visit (day 21). Similarly, *Salmonella* Derby and Ohio were recovered from manure samples in NCF 5. We did not recover any *Salmonella* in the field before manure application on this farm. After two hour of land application, we detected *S.* Derby and *S.* Ohio from the same soil samples. However, they were not recovered in the next sequential visits on days 7, 14 and 21.

2.4.2 Identification and distribution of *Salmonella* serotypes

The 189 *Salmonella* isolates were represented by 18 different serotypes (Table 2.1; Appendix 3). The serotypes detected in one state were not reported from the other. Three *Salmonella* serotypes were identified in IA, including *Salmonella* Anatum (6.88%), *S.* Litchfield (3.70%), and *S.* Infantis (0.53%). We observed a wider distribution of *Salmonella* serotypes in NC predominantly represented by *S.* Typhimurium var5- (22.22%), *S.* Senftenberg (14.81%), *S.* Rissen (8.99%) and *S.* Muenster (8.47%). Majority of the NC swine lagoon samples were represented by *S.* Rissen. There was a wide distribution of serotypes detected within each NC farm and some serotypes were detected in more than one farm including Worthington, Johannesburg, Derby, Rissen, and Typhimurium var5- (Table 2.1). We observed persistence of specific *Salmonella* serotypes throughout a farm in all the samples collected after manure application. This was seen in case of *S.* Altona and *S.* Muenster serotypes in samplings conducted on NCF 1, while *S.* Typhimurium var5- and *S.* Johannesburg were prevalent in samples collected from NCF 3. In contrast, *S.* Senftenberg

was isolated from NCF 4 throughout the sampling period at all stages, including from soil before manure application.

2.4.3 Antimicrobial resistance profile of *Salmonella*

A total of 189 *Salmonella* isolates (NC = 168, IA = 21) were tested for AST using Sensititre® containing a panel of 15 antimicrobial drugs. A squashtogram was created to represent the MIC distribution and AMR profile of *Salmonella* isolated in NC and IA swine farms (Table 2.2; Appendix 4). *Salmonella* isolates exhibited highest frequency of resistance to STR (88.36%) followed by FIS (67.2%) and TET (57.67%). A large proportion of the AMR Salmonella isolates were MDR (111/189; 58.73%), including a significantly higher number in NC (63.1%) than IA (23.81%) ($P = 0.001$). Only 8.47% of total isolates were pan-susceptible which was observed predominantly in serotype Anatum isolated from IA manure samples. The highest frequency of resistance in NC was exhibited to STR (89.29%), FIS (73.21%), and TET (63.69%) while in IA, the *Salmonella* isolates were predominantly resistant to STR (80.95%), AXO and XNL (23.81%), and FIS and FOX (19.05%) (Appendices 4-5). In addition, we observed that NC isolates also exhibited resistance to other aminoglycosides including KAN (47.02%), and GEN (17.26%). None of the *Salmonella* isolates were resistant to AZI, CIP, and NAL. The most frequent AMR patterns, associated serotypes, and their distributions are categorized in Table 2.3. AMP FIS KAN STR (n = 19) was the most common MDR pattern that was identified in NC from both lagoon and soil samples and was found to be significantly associated with *S.* Typhimurium var5- ($P<0.0001$; OR = 120.61). Another major MDR pattern associated with *S.* Typhimurium var5- was FIS KAN STR (n = 17) ($P<0.0001$; OR =1). This later pattern was only found in soil sample

from NC. *S.* Senftenberg (n = 28) and *S.* Worthington (n = 15) were the frequent serotypes isolated from NC and were also associated with MDR patterns (P<0.0001: $OR_{Senftenberg}$ = 215.28, $OR_{Worthington}$ = 15.9). The most common serotype found in NC lagoon (*S.* Rissen; n = 17) was associated with the STR TET pattern.

2.4.4 Pulse field gel electrophoresis (PFGE)

Genotypic characterization using PFGE with *Xba*I restriction enzyme generated on an average 10 to 18 DNA bands and distributed the *Salmonella* isolates (n = 189) into seven major clusters represented by NC (six clusters) and IA (one cluster) (Fig 2.5). Each individual major cluster was represented by *Salmonella* isolates belonging to the same serotype and were related to farm of origin. Distinct serotype distributions were detected in the different NC farms as exhibited in the three separate dendrograms that were created (Figs 2.2–2.4). *S.* Senftenberg (Cluster A) (Fig 2.2) isolated from multiple sampling points in NCF 4, including manure and day0 (after manure application), days7, 14 and 21 soil samples, had 100% similar PFGE profiles. In addition, all *S.* Senftenberg isolates in this cluster were MDR and shared the same R-pattern (FIS STR TET) highlighting the dissemination and persistence of this serotype after manure application based on phenotypic and genotypic characterization. Similarly, we detected genotypic similar *S.* Altona (Cluster B) isolated from NCF 1 soil at different time points (day0, day7, day14, and day21) (Fig 2.3). A single isolate from lagoon was grouped in this cluster and had similar PFGE pattern with another *S.* Altona isolated from soil on day7. The isolates in this cluster were predominantly pan-susceptible. Finally, *S.* Rissen (Cluster F) from NCF 6 on day 0 from lagoon and soil after manure application were genotypically identical (Fig 2.4). We detected two clusters that were composed of *Salmonella*

isolated only from soil and not from manure (Fig 2.5). This includes Cluster D (serotype Litchfield; n = 5) and E (*S.* Typhimurium var5-; n = 23). We used a 90% cut-off genotypic similarity to create the different clusters for our analysis. Using a less stringent cut-off value would have created bigger clusters, however we used a conservative approach. Seventy seven isolates did not cluster in any specific group and were represented as singletons.

2.5 Discussion

To date, no comprehensive research has been conducted on commercial swine farms to study the dissemination and persistence of AMR *Salmonella* from swine manure systems to soil environment after land application. The main objective of this study was to determine whether swine manure application in the farm environment leads to dissemination of *Salmonella*. In addition, we wanted to determine the impact of geographic location and the distinct waste management systems in the two states on *Salmonella* prevalence. Swine farms sampled in NC used a lagoon system for swine manure disposal while the farms covered in IA typically used a deep-pit storage system to store manure before applied on agricultural lands. In our study, *Salmonella* prevalence was significantly higher in manure samples than in soil samples ($P < 0.0001$).We observed a decrease in prevalence of *Salmonella* at different time points of sampling date (from day 0 to day 21), except in NCF 3 where the prevalence increased between day7-day14 after manure application. Based on our records, this farm had experienced a heavy rainfall event before sampling on day 14. Studies have explored the association between rainfall and microbial contamination where heavy rainfall events before dry spells have shown to assist in pathogen dissemination (41–42).We observed that *Salmonella* can persist on land at least 3 weeks after swine manure application. Factors

contributing to the survival of *Salmonella* in soil include temperature, moisture, soil type, plants, UV light, and soil organisms (43). In contrast to our study, *Salmonella* was rarely isolated in the soil samples before manure application in both states. We isolated *Salmonella* from a single farm in IA while all six farms in NC tested positive. *Salmonella* prevalence was found to be dependent on the swine manure storage system (lagoon or pit). Further, the subsequent dissemination and persistence in the environment was found to be dependent on the manure application method (spraying or injection) being employed on the farm. Once disseminated, *Salmonella* can persist in the environment for a significant amount of time depending on the geographic location and weather prevalent in the region. *Salmonella* spp. has been reported to survive in manure-amended soils from 2–3 until 332 days (13, 43–45). Even in the absence of active fertilization, *Salmonella* has been isolated ubiquitously in environmental soil samples collected from agricultural and recreational areas (46, 47). It is quite possible that the soil characteristics and weather conditions may have a direct impact on pathogen survival than in the manner the manure was applied.

Multiple *Salmonella* serotypes were identified in our study and none of the serotypes detected in one state were reported from the other. The most common serotypes detected in NC farms environment were Typhimurium var5-, Senftenberg, and Rissen. Previous study in NC reported that the predominant serotypes isolated from swine farms were Typhimurium followed by Infantis, Derby, and Anatum (34). In contrast, *S*. Anatum was not identified in NC, but was the predominant serovar in IA pit and soil samples. Abley et al. (48) reported that the top three *Salmonella* serotypes over the leading swine producing states (Iowa, North Carolina, and Minnesota) were Typhimurium (42%), Derby (25%) and Adelaide (5%). These

reports are in agreement with the CDC annual surveillance data which reports the most frequent *Salmonella* serotypes from porcine source are *S.* Typhimurium, *S.* Derby, *S.* Agona, *S.* Infantis, and *S.* Heidelberg (49). We observed that *S.* Rissen was one of the most common serotypes in NC farms especially in the manure samples from lagoon. This serotype was not well known until the outbreak in California 2008–2009 which resulted from the consumption of white ground pepper imported from Asia (49–51). *S.* Rissen is the most frequent and dominant serotype presented in south-east Asian countries especially in swine herds and retail pork and is reported to be MDR (52–55). In contrast, *S.* Rissen was identified for the first time in swine herds and environment in NC in the year 2009 (34).

Salmonella isolates from our study in both NC and IA were resistant to various classes of antimicrobials including streptomycin (88.36%), sulfisoxazole (67.2%), tetracycline (57.67%), kanamycin (43.39%) while 58.73% were MDR. These results are in accordance with previous studies in swine production where tetracycline resistant *Salmonella* were reported in the highest frequency followed by streptomycin and sulfisoxazole (34, 48, 56). Heuer et al. (57) documented that the antimicrobial compounds of sulfamethazine, tetracycline, chlortetracycline and tylosin used in farms were recovered from spread manure to agricultural soils. This evidence supports the association between agricultural antimicrobial use and its resistance (31). The excessive or inappropriate applications of antimicrobials in food animals described as therapeutic, prophylactic, and sub-therapeutic uses are considered to be the key of antimicrobial resistance problem (31). Department of Agriculture (58) reported that approximately 88% of commercial swine farms in US used antimicrobials, frequently tetracycline or tylosin in their feed for disease prevention and

growth promotion purposes. Most antimicrobial uses in farms need prescriptions from veterinarians, even though the particular treatment decisions are administrated by the farmworkers (31). The most common MDR-patterns in our study were FIS STR TET (16.4%), FIS GEN KAN STR TET (12.7%), and AMP FIS KAN STR (10.58%) which were significantly associated with serotypes Senftenberg, Worthington, and Typhimurium var5-, respectively. *S*. Typhimurium is common in swine production and mostly observed as MDR (34, 48, 59). The high frequency of resistance to different antimicrobials in *Salmonella* isolates (91.53%) recovered in our study from manure and soil samples is concerning. This is especially true in our study since majority of the soil samples before manure application were negative for the pathogen. Therefore, based on our study results we state that swine manure application does leads to dissemination of AMR *Salmonella* to the farm environment. However, it is important to note that our study was conducted on limited number of commercial swine farms and has limited internal and external validity.

Genotypic characterization by PFGE distributed the 189 *Salmonella* isolates into seven major clusters based on serotype and has been reported previously (48, 52, 60). The presence of clonal *Salmonella* isolates with identical phenotypic R-patterns suggests an epidemiological link between *Salmonella* recovered from manure and soil at different time points. The PFGE profiles (Figs 2.2–2.5) confirms the finding that *Salmonella* can be disseminated from manure use and persist in the environment at least 3 weeks after land application which is in accordance with serotype distribution in each farm at different time points (Table 2.1). Bech et al. (12) reported detecting *Salmonella* up to a month after application in loamy soil under cold and moist conditions. *S*. Typhimurium has been shown

to persist in pig slurry applied to a Danish field up to 14 days (61) while *E. coli* has been detected on day 21 after manure amendment (27). Studies have reported the presence of pathogens, AMR genes, and antimicrobial residues in lagoons and on lands after exposed to the swine manure (8, 10, 62, 63). According to the study results, we observed that *Salmonella* presents in swine manure, when spread on land, can persist for at least 21 days in soil (the longest period that could be detected in our study). However, the period of persistence varied among farms and states of origin. It is also important to highlight that not all clusters were represented by *Salmonella* isolates from soil and manure. Cluster D and E consisted of *Salmonella* serotypes that were isolated only from the soil. Clearly it is possible that these specific strains representing different *Salmonella* serotypes were already present in the soil even before manure application.

2.6 Conclusion

Based on phenotypic and genotypic characterization, our study highlighted the potential dissemination of AMR *Salmonella* after swine manure application in the environment. *Salmonella* present in swine manure, after spread on land, was able to persist in soil for at least 21 days in three out of the seven farms that were positive for the pathogen. The persistence of *Salmonella* in manure amended soils can have important public health implications. The dissemination of AMR *Salmonella* was dependent on the geographic location of the farm, waste storage system and on the specific manure application approach employed by the farm management. We acknowledge that the soil characteristics and existing weather conditions may have an impact on *Salmonella* survivability. It will be

important to conduct future comprehensive longitudinal and quantitative based study to study the dissemination of AMR pathogens from livestock manure application in the environment.

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Table 2.1 Distribution of *Salmonella* serotypes by farms at different time points following manure application

Farm (n=189)	Day 0[c]			Day 7[b]	Day 14[b]	Day 21[b]
	Lagoon / Pit[a]	**Before[b]**	**After[b]**			
NCF 1 (n=47)	Altona (1; 2.13%)		Altona (5; 10.64%)	Altona (4; 8.51%)	Altona (2; 4.26%)	Altona (2; 4.26%)
	Mbandaka (1; 2.13%)		Mbandaka (1; 2.13%)	Mbandaka (1; 2.18%)		
	Muenster (5; 10.64%)		Muenster (5; 10.64%)	Muenster (3; 6.38%)	Muenster (3; 6.38%)	
	Uganda (2; 4.26%)		Uganda (1; 2.13%)	Uganda (1; 2.13%)		
	Worthington (1; 2.18%)		Worthington (8; 17.02%)	Worthington (1; 2.13%)		
NCF 2 (n=4)	Derby (2; 50%)					
	Rough_O:z10:e,n,z15 (1; 25%)					
			Johannesburg (1; 25%)			
NCF 3 (n=59)				Derby (1; 1.69%)	Derby (3; 5.08%)	Derby (1; 1.69%)
	Johannesburg (4; 6.78%)			Johannesburg (2; 3.39%)	Johannesburg (1; 1.69%)	
	Rissen (1; 1.69%)					
	Typhimurium var5- (2; 3.39%)			Typhimurium var5- (15; 25.42%)	Typhimurium var5- (16; 27.12%)	Typhimurium var5- (8; 13.56%)
			Worthington (5; 8.47%)			
NCF 4 (n=30)				6,7:-:e,n,z15 (1; 3.33%)		
						Mbandaka (1; 3.33%)
	Senftenberg (9; 30%)	Senftenberg (1; 3.33%)	Senftenberg (10; 33.33%)	Senftenberg (6; 20%)	Senftenberg (1; 3.33%)	Senftenberg (1; 3.33%)

Table 2.1 Continued

Farm (n=189)	Day 0[c]			Day 7[b]	Day 14[b]	Day 21[b]
	Lagoon / Pit[a]	**Before[b]**	**After[b]**			
NCF 5 (n=11)	Derby (1; 9.09%) Ohio (3; 27.27%) Typhimurium var5- (1; 9.09%)		Derby (1; 9.09%) Ohio (4; 36.36%)	Ouakam (1; 9.09%)		
NCF 6 (n=17)	Rissen (9; 52.94%)		4,12:i:- (1; 5.88%) Rissen (7; 41.18%)			
IAF 6 (n=21)	Anatum (10; 47.62%)		Anatum (2; 9.52%) Infantis (1; 4.76%) Litchfield (6; 28.57%)	Anatum (1; 4.76%) Litchfield (1; 4.76%)		

[a] Manure (NCF 1-6) in NC is stored in lagoons while in IA (IAF 6), it is stored in the form of slurry in pits.

[b] Soil samples

[c] The percentage was calculated within each commercial swine farm.

Table 2.2 Comparison of resistance and MIC distribution for *Salmonella* isolated in North Carolina and Iowa (NC = 168; IA = 21)

AM[a]	State	%R[b]	Distribution of MICs in µg/mL (%)															
			0.015	**0.03**	**0.06**	**0.125**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**
AMP	NC	25.60						51.8	1.8	13.7	3.6	1.2	2.4	2.4	23.2			
	IA	14.29						33.3	0.0	42.9	4.8	0.0	4.8	0.0	14.3			
AUG2	NC	7.14						61.3	0.0	40.7	1.2	1.2	24.4	4.2	3.6			
	IA	14.29						66.7	0.0	14.3	0.0	0.0	4.8	4.8	9.5			
AXO	NC	5.36					91.1	2.4	0.0	1.2	0.0	1.2	3.6	0.6	0.0			
	IA	23.81					76.2	0.0	0.0	0.0	0.0	0.0	14.3	4.7	4.7			
AZI	NC	0				0.0	0.0	0.0	3.0	18.5	57.1	20.2	1.2					
	IA	0				0.0	0.0	0.0	0.0	0.0	42.9	57.1	0.0					
CHL	NC	1.19								0.0	17.9	79.8	1.2	1.2				
	IA	0								0.0	9.5	85.7	4.8	0.0				
CIP	NC	0	79.2	19.0	1.2	0.0	0.6	0.0	0.0	0.0	0.0							
	IA	0	47.6	47.6	4.8	0.0	0.0	0.0	0.0	0.0	0.0							
FIS	NC	73.21											3.0	2.4	14.9	6.0	0.6	73.2
	IA	19.05											0.0	9.5	23.8	14.3	33.3	19.0
FOX	NC	6.55						0.0	0.6	18.5	66.7	5.4	2.4	4.2	2.4			
	IA	19.05						0.0	0.0	19.0	52.4	4.8	4.8	9.5	9.5			
GEN	NC	17.26					1.8	54.8	25.0	1.2	0.0	0.0	1.2	16.1				
	IA	0					28.6	42.9	28.6	0.0	0.0	0.0	0.0					
KAN	NC	47.02									51.8	0.0	0.0	1.2	3.6	43.5		
	IA	14.29									81.0	0.0	0.0	4.8	0.0	14.3		
NAL	NC	0						0.0	0.0	42.3	56.0	1.2	0.6	0.0				
	IA	0						0.0	9.5	0.0	85.7	4.8	0.0	0.0				
STR	NC	89.29												10.7	13.1	76.2		
	IA	80.95												19.0	0.0	81.0		
SXT	NC	5.36			71.4	1.2	14.3	7.7	0.0	0.0	0.0	5.4						
	IA	4.76			95.2	0.0	0.0	0.0	0.0	0.0	0.0	4.8						

Table 2.2 Continued

AM[a]	State	%R[b]	Distribution of MICs in µg/mL (%)														
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
XNL	NC	5.36				0.0	0.0	20.2	72.6	0.0	1.8	5.4						
	IA	23.81				0.0	0.0	0.0	71.4	4.8	0.0	23.8						
TET	NC	63.69								19.6	16.7	0.0	1.2	1.2	61.3			
	IA	9.52								90.5	0.0	0.0	4.8	0.0	4.8			

The vertical bars indicate the breakpoints for resistance.

The unshaded areas indicate the range of dilutions tested for each antimicrobials. Shaded areas fall outside the range of tested concentrations. The vertical bars indicate the breakpoints for resistance.

[a] amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 µg/ml), ampicilin (AMP; 1-32 µg/ml), azithromycin (AZI; 0.12-16 µg/ml), cefoxitin (FOX; 0.5-32 µg/ml), ceftiofur (XNL; 0.12-8 µg/ml), ceftriaxone (AXO; 0.25-64 µg/ml), chloramphenicol (CHL; 2-32 µg/ml), ciprofloxacin (CIP; 0.015-4 µg/ml), gentamicin (GEN; 0.25-16 µg/ml), kanamycin (KAN; 8-64 µg/ml), nalidixic acid (NAL; 0.5-32 µg/ml), streptomycin (STR; 32-64 µg/ml), sulfisoxazole (FIS; 16-256 µg/ml), trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 µg/ml), and tetracycline (TET; 4-32 µg/ml)

[b] Percent resistant isolates to each antimicrobial in a state.

Table 2.3 Distribution of *Salmonella* serotypes associated with predominant R-patterns

***Salmonella* serotypes (n)**	**Predominant patterns[a] (n)**	**Manure n (%)[b]**	**Soil n (%)[b]**
Typhimurium var5-[1] (42)	AMP FIS KAN STR (19)	2 (10.53)	17 (89.47)
	FIS KAN STR (17)	0	17 (100)
	AMP AUG2 FIS KAN STR (2)	0	2 (100)
	FIS KAN STR TET (2)	0	2 (100)
	AMP CHL FIS KAN STR TET (1)	1 (100)	0
	AMP FIS STR (1)	0	1 (100)
Senftenberg[1] (28)	FIS STR TET (25)	7 (28.0)	18 (72.0)
	AMP FIS STR TET (2)	2 (100)	0
	AMP AUG2 FIS FOX KAN STR TET (1)	0	1 (100)
Worthington[1] (15)	FIS GEN KAN STR TET (9)	0	9 (100)
	STR TET (5)	0	5 (100)
	FIS GEN KAN STR SXT TET (1)	0	1 (100)
Rissen[1] (17)	STR TET (15)	9 (60)	6 (40)
	AMP CHL FIS STR SXT TET (1)	1 (100)	0
	AMP STR TET (1)	0	1 (100)
Anatum[2] (13)	Pan-susceptible (3)	3 (100)	0
	STR (3)	1 (33.33)	2 (66.67)
	AMP AUG2 AXO FOX KAN STR XNL (2)	2 (100)	0
	FIS STR (2)	1 (50)	1 (50)
	AMP AXO FIS STR XNL (1)	1 (100)	0
	AUG2 AXO FOX KAN STR XNL (1)	1 (100)	0
	AXO FOX XNL (1)	1 (100)	0

[a]ampicillin (AMP), amoxicillin/clavulanic acid (AUG2), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), kanamycin (KAN), streptomycin (STR), sulfisoxazole (FIS), trimetroprim/sulfamethoxazole (SXT), and tetracycline (TET)

[b]number of isolates (percent resistant to a specific R-pattern)

[1]*Salmonella* serotypes isolated from commercial swine farms in North Carolina.

[2]*Salmonella* serotype isolated from commercial swine farm in Iowa.

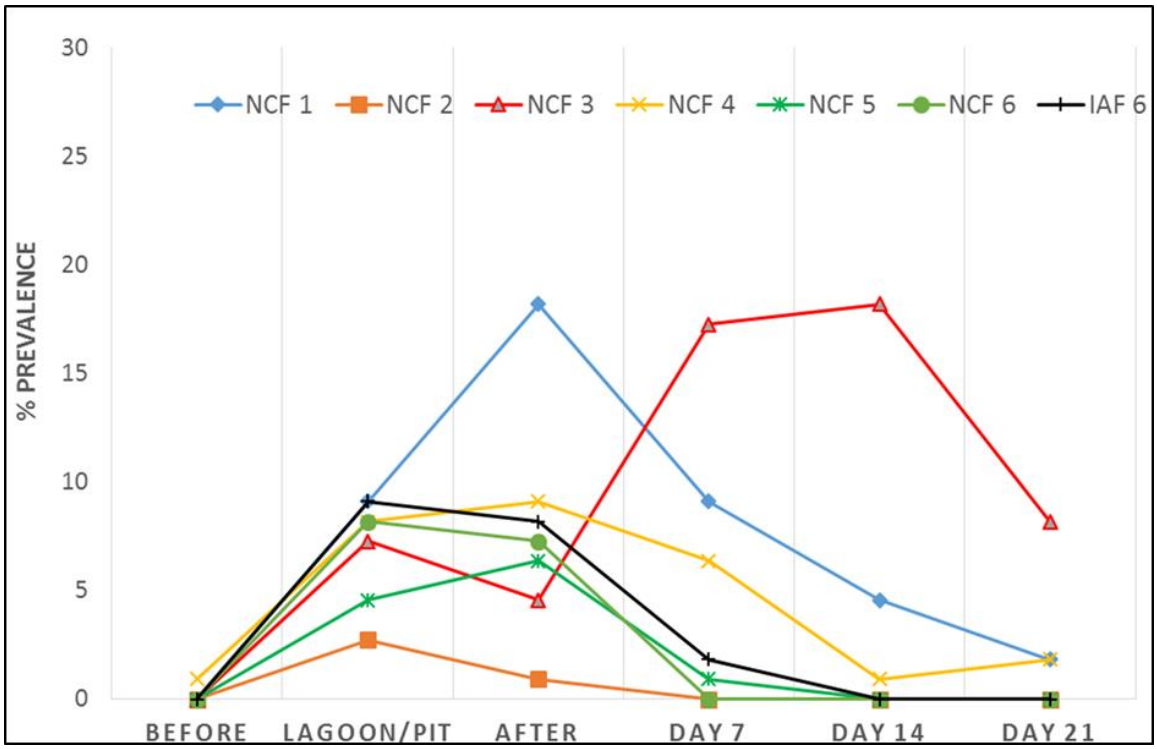


Figure 2.1 *Salmonella* prevalence among North Carolina samples (NCF 1- NCF 6) and Iowa samples (IAF 6) at different time points.

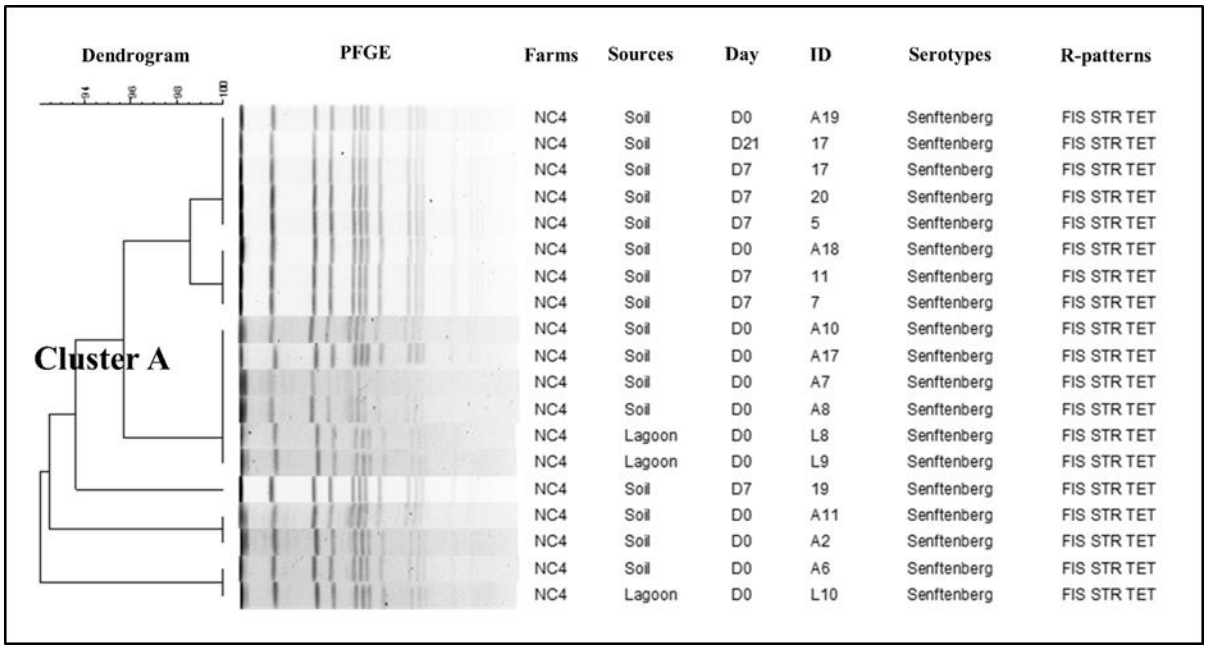


Figure 2.2 Phylogenetic analysis representing PFGE-*Xba*I with antimicrobial resistance patterns of *Salmonella* Senftenberg isolated from NCF 4 at 90% cut-off genetic similarity (cluster A).

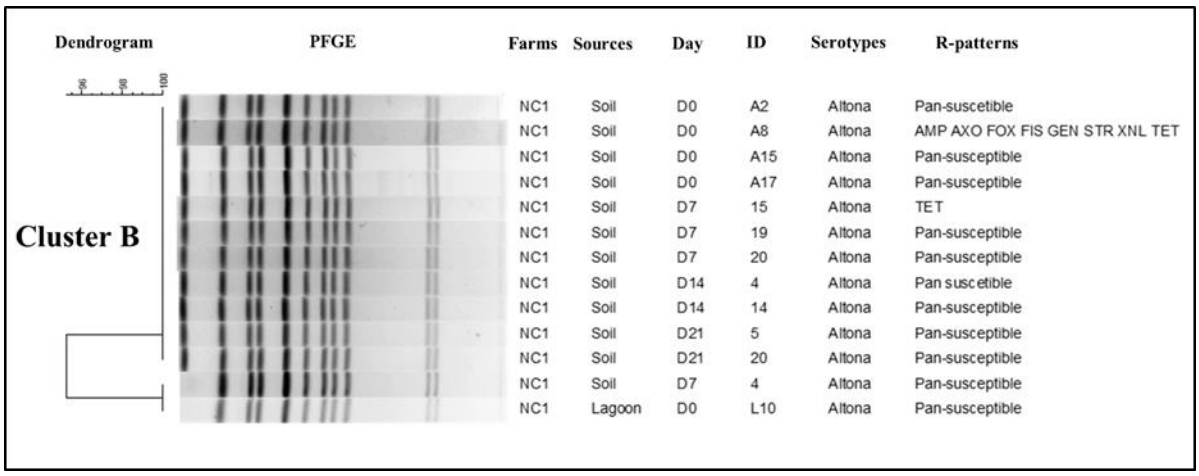


Figure 2.3 Phylogenetic analysis representing PFGE-*Xba*I with antimicrobial resistance patterns of *Salmonella* Altona isolated from NCF 1 at 90% cut-off genetic similarity (cluster B).

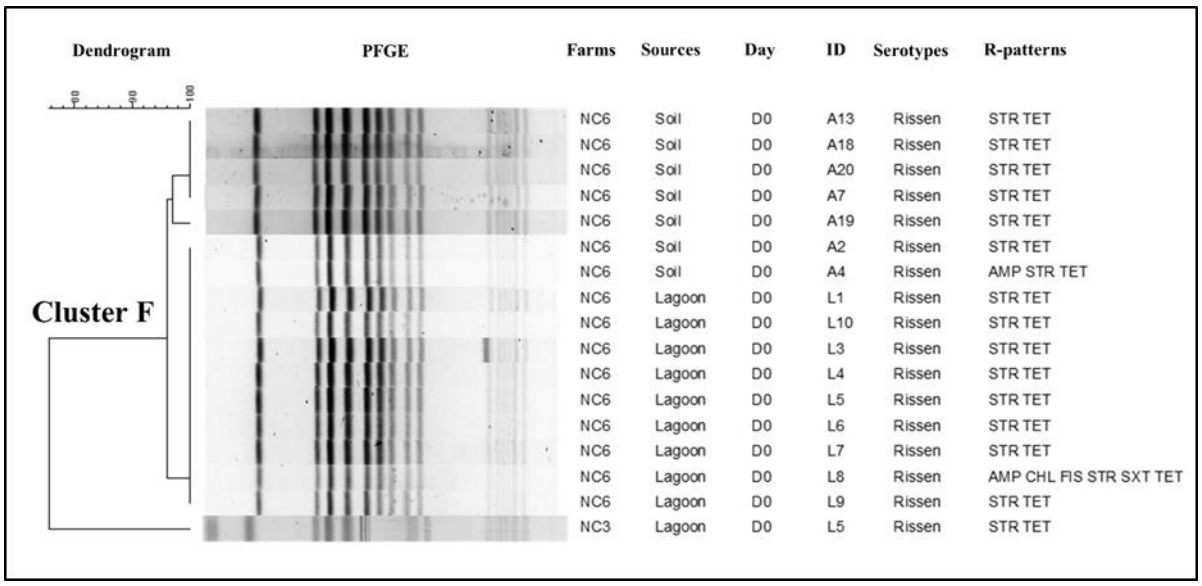
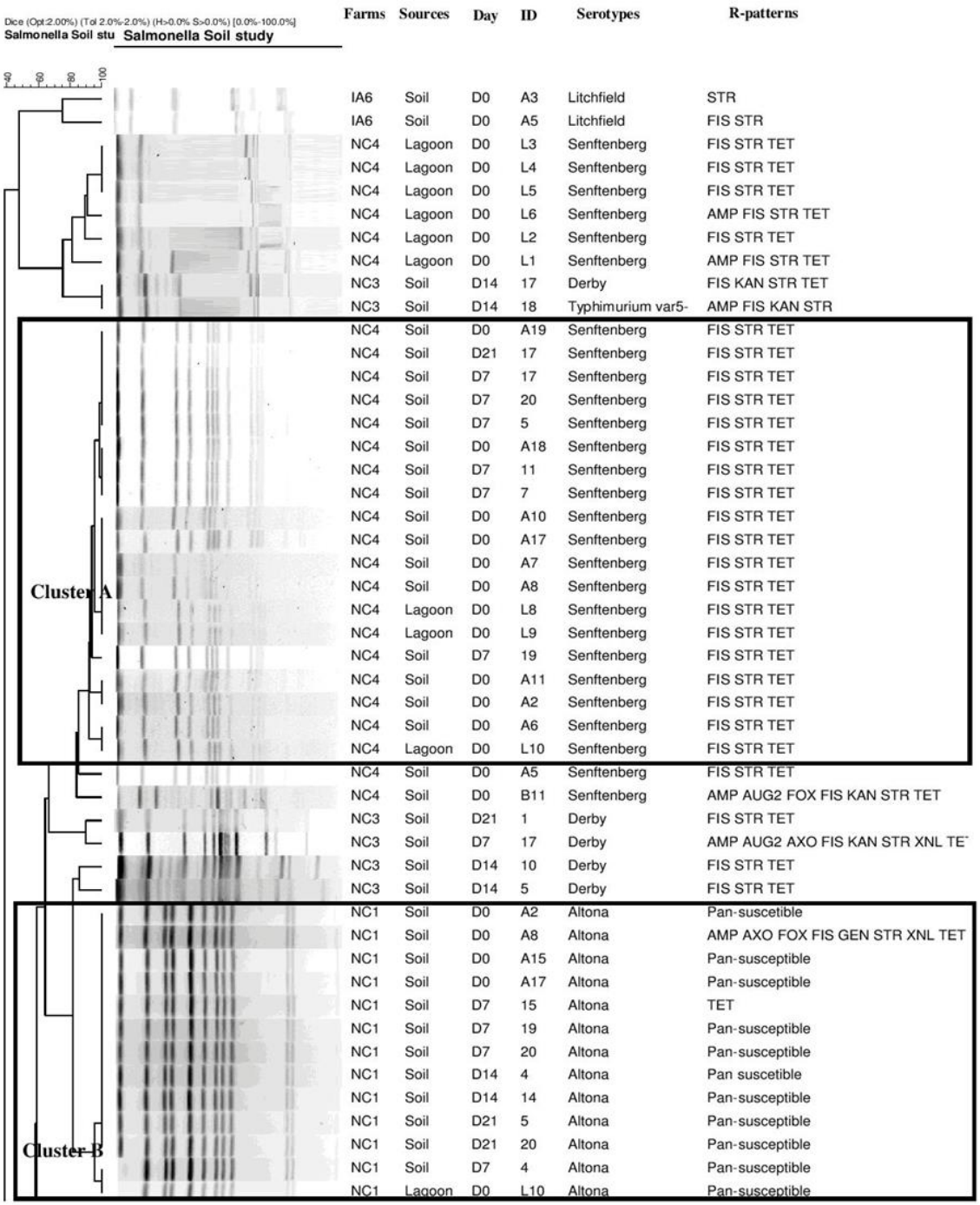
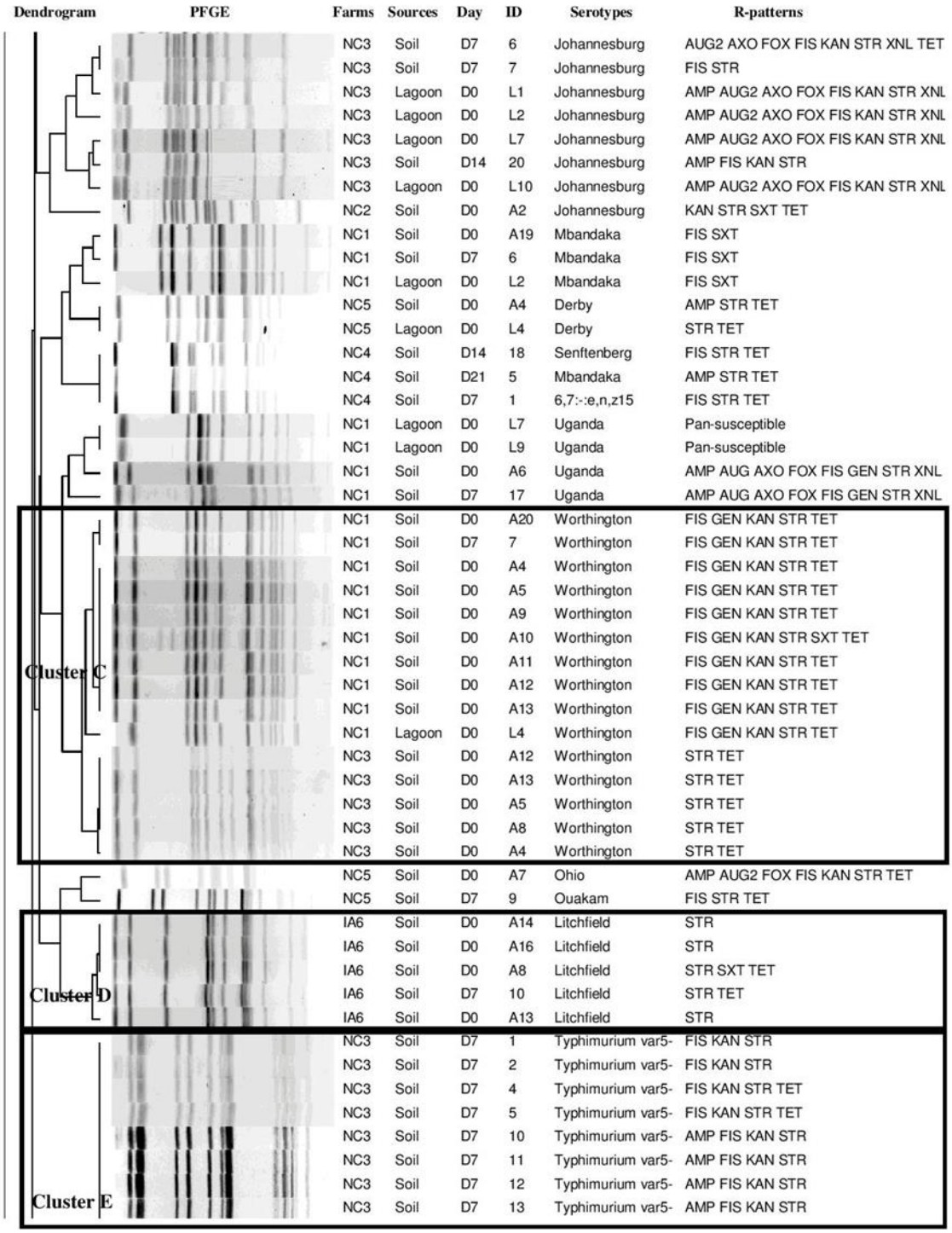


Figure 2.4 Phylogenetic analysis representing PFGE-*Xba*I with antimicrobial resistance patterns of *Salmonella* Rissen isolated from NCF 3 and 6 at 90% cut-off genetic similarity (cluster F).

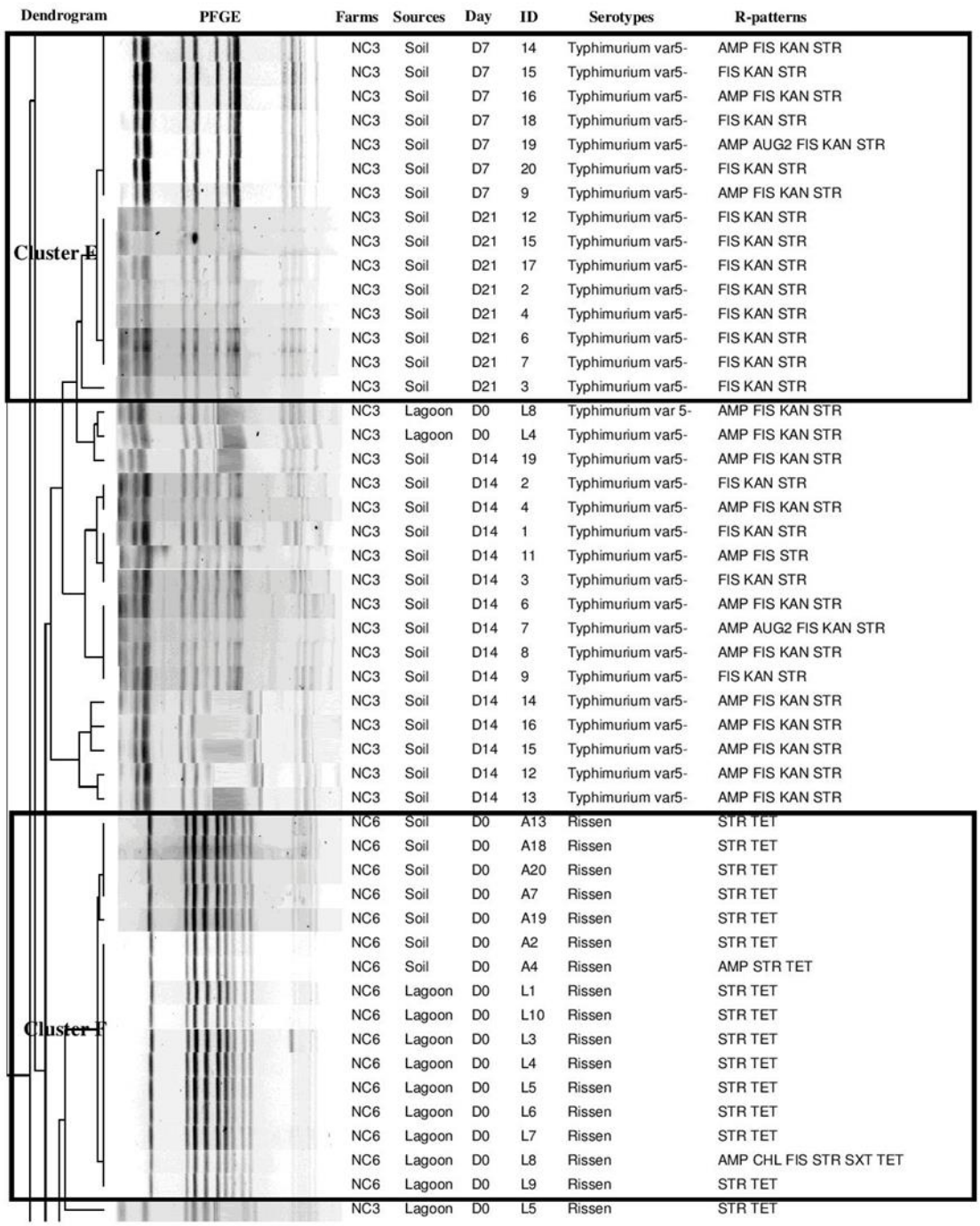
Figure 2.5 Phylogenetic analysis representing PFGE-*Xba*I with antimicrobial resistant patterns of *Salmonella* isolated from NC and IA commercial swine farms at 90% cut-off genotypic similarity; cluster A-G (Supplement).



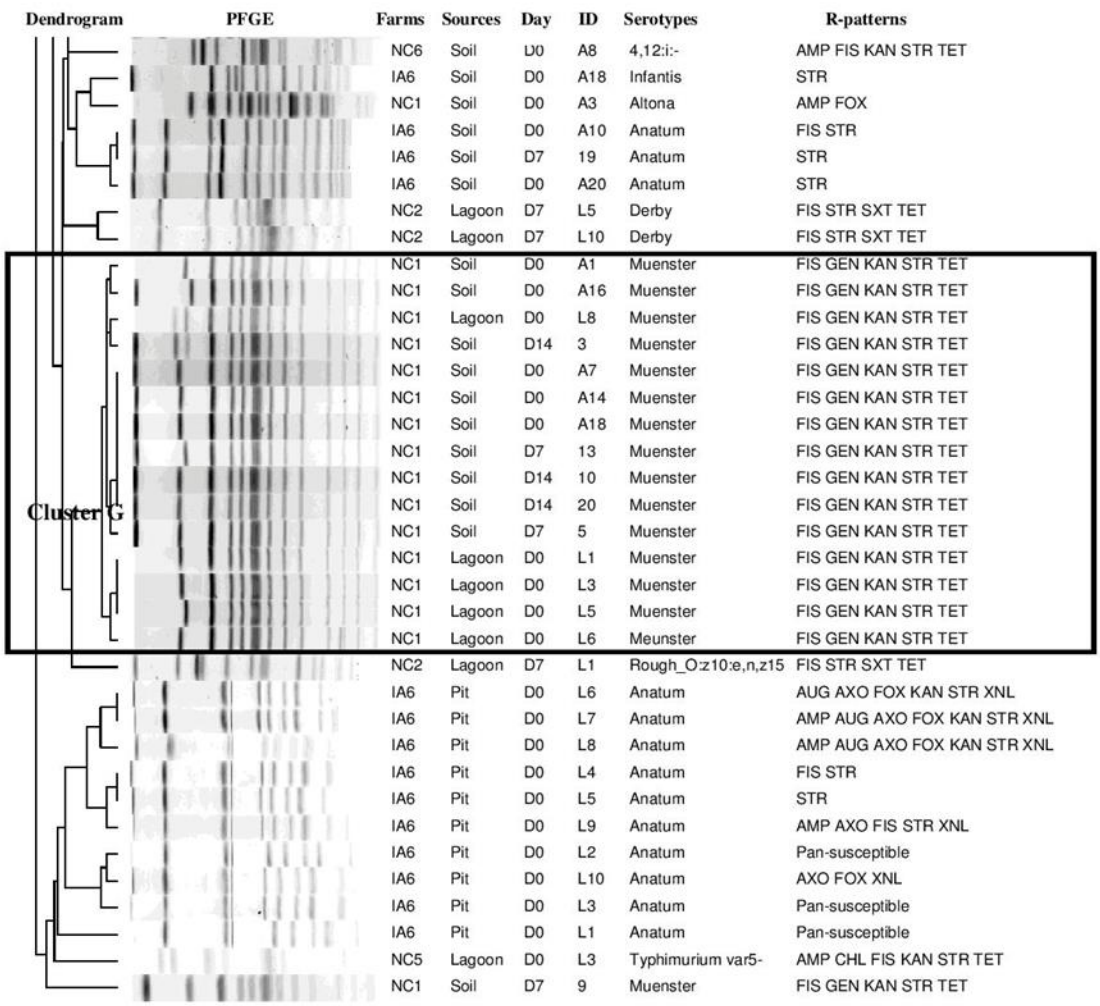
Farms	Sources	Day	ID	Serotypes	R-patterns
IA6	Soil	D0	A3	Litchfield	STR
IA6	Soil	D0	A5	Litchfield	FIS STR
NC4	Lagoon	D0	L3	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L4	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L5	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L6	Senftenberg	AMP FIS STR TET
NC4	Lagoon	D0	L2	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L1	Senftenberg	AMP FIS STR TET
NC3	Soil	D14	17	Derby	FIS KAN STR TET
NC3	Soil	D14	18	Typhimurium var5-	AMP FIS KAN STR
NC4	Soil	D0	A19	Senftenberg	FIS STR TET
NC4	Soil	D21	17	Senftenberg	FIS STR TET
NC4	Soil	D7	17	Senftenberg	FIS STR TET
NC4	Soil	D7	20	Senftenberg	FIS STR TET
NC4	Soil	D7	5	Senftenberg	FIS STR TET
NC4	Soil	D0	A18	Senftenberg	FIS STR TET
NC4	Soil	D7	11	Senftenberg	FIS STR TET
NC4	Soil	D7	7	Senftenberg	FIS STR TET
NC4	Soil	D0	A10	Senftenberg	FIS STR TET
NC4	Soil	D0	A17	Senftenberg	FIS STR TET
NC4	Soil	D0	A7	Senftenberg	FIS STR TET
NC4	Soil	D0	A8	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L8	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L9	Senftenberg	FIS STR TET
NC4	Soil	D7	19	Senftenberg	FIS STR TET
NC4	Soil	D0	A11	Senftenberg	FIS STR TET
NC4	Soil	D0	A2	Senftenberg	FIS STR TET
NC4	Soil	D0	A6	Senftenberg	FIS STR TET
NC4	Lagoon	D0	L10	Senftenberg	FIS STR TET
NC4	Soil	D0	A5	Senftenberg	FIS STR TET
NC4	Soil	D0	B11	Senftenberg	AMP AUG2 FOX FIS KAN STR TET
NC3	Soil	D21	1	Derby	FIS STR TET
NC3	Soil	D7	17	Derby	AMP AUG2 AXO FIS KAN STR XNL TE
NC3	Soil	D14	10	Derby	FIS STR TET
NC3	Soil	D14	5	Derby	FIS STR TET
NC1	Soil	D0	A2	Altona	Pan-suscetible
NC1	Soil	D0	A8	Altona	AMP AXO FOX FIS GEN STR XNL TET
NC1	Soil	D0	A15	Altona	Pan-susceptible
NC1	Soil	D0	A17	Altona	Pan-susceptible
NC1	Soil	D7	15	Altona	TET
NC1	Soil	D7	19	Altona	Pan-susceptible
NC1	Soil	D7	20	Altona	Pan-susceptible
NC1	Soil	D14	4	Altona	Pan suscetible
NC1	Soil	D14	14	Altona	Pan-susceptible
NC1	Soil	D21	5	Altona	Pan-susceptible
NC1	Soil	D21	20	Altona	Pan-susceptible
NC1	Soil	D7	4	Altona	Pan-susceptible
NC1	Lagoon	D0	L10	Altona	Pan-susceptible



Dendrogram	PFGE	Farms	Sources	Day	ID	Serotypes	R-patterns
		NC3	Soil	D7	6	Johannesburg	AUG2 AXO FOX FIS KAN STR XNL TET
		NC3	Soil	D7	7	Johannesburg	FIS STR
		NC3	Lagoon	D0	L1	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
		NC3	Lagoon	D0	L2	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
		NC3	Lagoon	D0	L7	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
		NC3	Soil	D14	20	Johannesburg	AMP FIS KAN STR
		NC3	Lagoon	D0	L10	Johannesburg	AMP AUG2 AXO FOX FIS KAN STR XNL
		NC2	Soil	D0	A2	Johannesburg	KAN STR SXT TET
		NC1	Soil	D0	A19	Mbandaka	FIS SXT
		NC1	Soil	D7	6	Mbandaka	FIS SXT
		NC1	Lagoon	D0	L2	Mbandaka	FIS SXT
		NC5	Soil	D0	A4	Derby	AMP STR TET
		NC5	Lagoon	D0	L4	Derby	STR TET
		NC4	Soil	D14	18	Senftenberg	FIS STR TET
		NC4	Soil	D21	5	Mbandaka	AMP STR TET
		NC4	Soil	D7	1	6,7:-:e,n,z15	FIS STR TET
		NC1	Lagoon	D0	L7	Uganda	Pan-susceptible
		NC1	Lagoon	D0	L9	Uganda	Pan-susceptible
		NC1	Soil	D0	A6	Uganda	AMP AUG AXO FOX FIS GEN STR XNL
		NC1	Soil	D7	17	Uganda	AMP AUG AXO FOX FIS GEN STR XNL
Cluster C		NC1	Soil	D0	A20	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D7	7	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A4	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A5	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A9	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A10	Worthington	FIS GEN KAN STR SXT TET
		NC1	Soil	D0	A11	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A12	Worthington	FIS GEN KAN STR TET
		NC1	Soil	D0	A13	Worthington	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L4	Worthington	FIS GEN KAN STR TET
		NC3	Soil	D0	A12	Worthington	STR TET
		NC3	Soil	D0	A13	Worthington	STR TET
		NC3	Soil	D0	A5	Worthington	STR TET
		NC3	Soil	D0	A8	Worthington	STR TET
		NC3	Soil	D0	A4	Worthington	STR TET
		NC5	Soil	D0	A7	Ohio	AMP AUG2 FOX FIS KAN STR TET
		NC5	Soil	D7	9	Ouakam	FIS STR TET
Cluster D		IA6	Soil	D0	A14	Litchfield	STR
		IA6	Soil	D0	A16	Litchfield	STR
		IA6	Soil	D0	A8	Litchfield	STR SXT TET
		IA6	Soil	D7	10	Litchfield	STR TET
		IA6	Soil	D0	A13	Litchfield	STR
Cluster E		NC3	Soil	D7	1	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D7	2	Typhimurium var5-	FIS KAN STR
		NC3	Soil	D7	4	Typhimurium var5-	FIS KAN STR TET
		NC3	Soil	D7	5	Typhimurium var5-	FIS KAN STR TET
		NC3	Soil	D7	10	Typhimurium var5-	AMP FIS KAN STR
		NC3	Soil	D7	11	Typhimurium var5-	AMP FIS KAN STR
		NC3	Soil	D7	12	Typhimurium var5-	AMP FIS KAN STR
		NC3	Soil	D7	13	Typhimurium var5-	AMP FIS KAN STR



Farms	Sources	Day	ID	Serotypes	R-patterns
NC3	Soil	D7	14	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D7	15	Typhimurium var5-	FIS KAN STR
NC3	Soil	D7	16	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D7	18	Typhimurium var5-	FIS KAN STR
NC3	Soil	D7	19	Typhimurium var5-	AMP AUG2 FIS KAN STR
NC3	Soil	D7	20	Typhimurium var5-	FIS KAN STR
NC3	Soil	D7	9	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D21	12	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	15	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	17	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	2	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	4	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	6	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	7	Typhimurium var5-	FIS KAN STR
NC3	Soil	D21	3	Typhimurium var5-	FIS KAN STR
NC3	Lagoon	D0	L8	Typhimurium var 5-	AMP FIS KAN STR
NC3	Lagoon	D0	L4	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	19	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	2	Typhimurium var5-	FIS KAN STR
NC3	Soil	D14	4	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	1	Typhimurium var5-	FIS KAN STR
NC3	Soil	D14	11	Typhimurium var5-	AMP FIS STR
NC3	Soil	D14	3	Typhimurium var5-	FIS KAN STR
NC3	Soil	D14	6	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	7	Typhimurium var5-	AMP AUG2 FIS KAN STR
NC3	Soil	D14	8	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	9	Typhimurium var5-	FIS KAN STR
NC3	Soil	D14	14	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	16	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	15	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	12	Typhimurium var5-	AMP FIS KAN STR
NC3	Soil	D14	13	Typhimurium var5-	AMP FIS KAN STR
NC6	Soil	D0	A13	Rissen	STR TET
NC6	Soil	D0	A18	Rissen	STR TET
NC6	Soil	D0	A20	Rissen	STR TET
NC6	Soil	D0	A7	Rissen	STR TET
NC6	Soil	D0	A19	Rissen	STR TET
NC6	Soil	D0	A2	Rissen	STR TET
NC6	Soil	D0	A4	Rissen	AMP STR TET
NC6	Lagoon	D0	L1	Rissen	STR TET
NC6	Lagoon	D0	L10	Rissen	STR TET
NC6	Lagoon	D0	L3	Rissen	STR TET
NC6	Lagoon	D0	L4	Rissen	STR TET
NC6	Lagoon	D0	L5	Rissen	STR TET
NC6	Lagoon	D0	L6	Rissen	STR TET
NC6	Lagoon	D0	L7	Rissen	STR TET
NC6	Lagoon	D0	L8	Rissen	AMP CHL FIS STR SXT TET
NC6	Lagoon	D0	L9	Rissen	STR TET
NC3	Lagoon	D0	L5	Rissen	STR TET



Dendrogram	PFGE	Farms	Sources	Day	ID	Serotypes	R-patterns
		NC6	Soil	D0	A8	4,12:i:-	AMP FIS KAN STR TET
		IA6	Soil	D0	A18	Infantis	STR
		NC1	Soil	D0	A3	Altona	AMP FOX
		IA6	Soil	D0	A10	Anatum	FIS STR
		IA6	Soil	D7	19	Anatum	STR
		IA6	Soil	D0	A20	Anatum	STR
		NC2	Lagoon	D7	L5	Derby	FIS STR SXT TET
		NC2	Lagoon	D7	L10	Derby	FIS STR SXT TET
		NC1	Soil	D0	A1	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A16	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L8	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D14	3	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A7	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A14	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D0	A18	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D7	13	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D14	10	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D14	20	Muenster	FIS GEN KAN STR TET
		NC1	Soil	D7	5	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L1	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L3	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L5	Muenster	FIS GEN KAN STR TET
		NC1	Lagoon	D0	L6	Meunster	FIS GEN KAN STR TET
		NC2	Lagoon	D7	L1	Rough_O:z10:e,n,z15	FIS STR SXT TET
		IA6	Pit	D0	L6	Anatum	AUG AXO FOX KAN STR XNL
		IA6	Pit	D0	L7	Anatum	AMP AUG AXO FOX KAN STR XNL
		IA6	Pit	D0	L8	Anatum	AMP AUG AXO FOX KAN STR XNL
		IA6	Pit	D0	L4	Anatum	FIS STR
		IA6	Pit	D0	L5	Anatum	STR
		IA6	Pit	D0	L9	Anatum	AMP AXO FIS STR XNL
		IA6	Pit	D0	L2	Anatum	Pan-susceptible
		IA6	Pit	D0	L10	Anatum	AXO FOX XNL
		IA6	Pit	D0	L3	Anatum	Pan-susceptible
		IA6	Pit	D0	L1	Anatum	Pan-susceptible
		NC5	Lagoon	D0	L3	Typhimurium var5-	AMP CHL FIS KAN STR TET
		NC1	Soil	D7	9	Muenster	FIS GEN KAN STR TET

3. CHAPTER III: Horizontal dissemination of antimicrobial resistance determinants in multiple *Salmonella* serotypes following isolation from the commercial swine operation environment after manure application.

Presented here is the manuscript titled "Horizontal dissemination of antimicrobial resistance determinants in multiple *Salmonella* serotypes following isolation from the commercial swine operation environment after manure application", published year 2017 in the journal of Applied and Environmental Microbiology, vol. 83(20), pgs. e01503-17. Additional data are presented in the Appendix.

3.1 Abstract

The aim of this study was to characterize the plasmids carrying antimicrobial resistance (AMR) determinants in multiple *Salmonella* serotypes recovered from the commercial swine farm environment after manure application on land. Manure and soil samples were collected on day 0 before and after manure application on six farms in North Carolina, and sequential soil samples were recollected on days 7, 14, and 21 from the same plots. All environmental samples were processed for *Salmonella*, and their plasmid contents were further characterized. A total of 14 isolates including *Salmonella enterica* serotypes Johannesburg (*n*=2), Ohio (*n*=2), Rissen (*n*=1), Typhimurium var5- (*n*=5), Worthington (*n*=3), and 4,12:i:- (*n*=1), representing different farms, were selected for plasmid analysis. Antimicrobial susceptibility testing was done by broth microdilution against a panel of 14 antimicrobials on the 14 confirmed transconjugants after conjugation assays. The plasmids were isolated by modified alkaline lysis, and PCR s were performed on purified plasmid DNA to identify the AMR determinants and the plasmid replicon types. The plasmids were

sequenced for further analysis and to compare profiles and create phylogenetic trees. A class 1 integron with an ANT(2")-Ia-*aad*A2 cassette was detected in the 50-kb IncN plasmids identified in *S.* Worthington isolates. We identified 100-kb and 90-kb IncI1 plasmids in *S.* Johannesburg and *S*. Rissen isolates carrying the *bla*CMY-2 and *tet*(A) genes, respectively. An identical 95-kb IncF plasmid was widely disseminated among the different serotypes and across different farms. Our study provides evidence on the importance of horizontal dissemination of resistance determinants through plasmids of multiple *Salmonella* serotypes distributed across commercial swine farms after manure application.

Importance

The horizontal gene transfer of antimicrobial resistance (AMR) determinants located on plasmids is considered to be the main reason for the rapid proliferation and spread of drug resistance. The deposition of manure generated in swine production systems into the environment is identified as a potential source of AMR dissemination. In this study, AMR gene-carrying plasmids were detected in multiple *Salmonella* serotypes across different commercial swine farms in North Carolina. The plasmid profiles were characterized based on *Salmonella* serotype donors and incompatibility (Inc) groups. We found that different Inc plasmids showed evidence of AMR gene transfer in multiple *Salmonella* serotypes. We detected an identical 95-kb plasmid that was widely distributed across swine farms in North Carolina. These conjugable resistance plasmids were able to persist on land after swine manure application. Our study provides strong evidence of AMR determinant dissemination present in plasmids of multiple *Salmonella* serotypes in the environment after manure application.

3.2 Introduction

The emergence of antimicrobial resistance (AMR) in bacterial pathogens has threatened the sustainability of an effective global public health response to infectious diseases (1, 2). There are major gaps in our understanding of AMR transmission within agricultural sites and the potential impacts on humans, animals, and the environment due to a lack of studies conducted on actual commercial food animal farms (3–5). A number of studies have documented the abundance of AMR pathogens associated with livestock production due to the intensive use of antimicrobials in animal husbandry practices for therapeutic and nontherapeutic purposes (6–9). However, there is limited knowledge about the effect of manure application on the spread of AMR pathogens and AMR genes by means of horizontal gene transfer (HGT), such as by plasmids, transposons, and integrons, in the environment (4, 10, 11). Exposure of bacterial pathogens to antimicrobials in the environment increases the evolution of resistance and has an influence on the abundance, distribution, and transfer of AMR genes into different bacterial species (9, 12). We recently reported the dissemination of AMR *Salmonella* isolates in manure from commercial swine farms that were able to persist on land for at least 21 days after manure application, and it was clearly observed that *Salmonella* bacteria were rarely present in the soil before the land application (13). Given the potential risk of disseminating AMR *Salmonella* bacteria into the environment during manure application, we further characterized the plasmids that were detected in the multiple *Salmonella* serotypes isolated in our previous study.

The dissemination of undesirable AMR genes in Gram-negative pathogenic bacteria has been mainly regarded as the acquisition of multiple plasmid-located AMR genes by HGT

(14, 15). Conjugation is considered the main mode of HGT of AMR genes among the *Enterobacteriaceae* family and helps to increase bacterial genetic diversity (16, 17). Plasmids conferring resistance have been identified as hindering antimicrobial therapy, including the use of extended-spectrum cephalosporins and fluoroquinolones, which are regarded as drugs of choice for bacterial infection in human clinical cases (14, 18, 19). Studies from several parts of the world have demonstrated the distribution of plasmids harboring extended-spectrum β-lactamase (ESBL) genes (*bla*CTX, *bla*SHV, *bla*CMY, and *bla*TEM) or *amp*C and plasmid-mediated quinolone resistance (PMQR) genes (*qnr*A, *qnr*B, and *qnr*S) in *Escherichia coli* and *Salmonella* among animal, human, and environmental sources (16, 20–22). The presence of plasmid-mediated transfer of a recently identified mobile colistin resistance gene (*mcr*-1) is another example of the threat posed to public health (23, 24). The comparative analysis of *mcr*-1-containing plasmids maintained in the *Enterobacteriaceae* family revealed that they are disseminated in a broad host range, including human, animal, and food sources, and are now being reported from different countries worldwide (25–27). Plasmids that confer resistance in carbapenem-resistant *Enterobacteriaceae* (CRE) pose an urgent threat to public health with their global expansion (28, 29). Mollenkopf et al. (30) reported that the CRE carrying *bla*IMP-27 plasmids were recovered from the environment of a swine production area in the United States. The farm environment is considered a potential reservoir of AMR *Salmonella* strains that probably exchange AMR determinants with humans and animals by plasmid horizontal transfer (13, 22, 31, 32).

The objective of this study was to determine and characterize the resistance plasmid profiles isolated from multiple AMR *Salmonella* serotypes recovered in manure and

environmental samples after land application of manure on commercial swine farms in North Carolina. To address this, we performed antimicrobial susceptibility testing (AST), plasmid replicon typing, conjugation assays, and plasmid sequencing to fully understand the role of these plasmids in transferring AMR determinants in the environment.

3.3 Results

3.3.1 *Salmonella* serotypes and plasmid characterization

A total of 14 different *Salmonella* serotypes isolated from commercial swine farms in North Carolina were selected to determine whether the AMR genes were located on transmissible plasmids. We also wanted to find out whether dissemination of AMR *Salmonella* bacteria through manure application assists in the transmission of genes via plasmids to other susceptible bacterial populations. *Salmonella* isolates collected from the swine farm environment after manure application were selected from each farm based on type of sample (lagoon and soil), sampling day, serotype, and resistance phenotype (Table 3.1). All 14 *Salmonella* donors harbored at least one large plasmid larger than 40 kb in size, and their plasmid profiles were dependent on farm origin and donor *Salmonella* serotype. PCR-based replicon typing (PBRT) revealed four plasmid replicons (FI, FII, I1, and N) among the 14 isolates carrying plasmids (Table 3.1). IncN plasmids (*n*=3) of 50 kb in size were found in *Salmonella enterica* serotype Worthington isolates from both lagoon and soil samples in North Carolina farm 1 (NCF1). In NCF3, 100-kb IncI1 (*n*=2) plasmids were isolated from *S. enterica* serotype Johannesburg while *S. enterica* serotype Typhimurium var5- was the predominant serotype at this farm and carried IncFII plasmids (*n*=4) of 95 kb in size. Furthermore, IncFII plasmids were also found in *S.* Typhimurium var5- from NCF5 and

4,12:i:- from NCF6. A single *S. enterica* serotype Rissen isolate from a lagoon sample in NCF6 carried an IncI1 plasmid of 90 kb in size. The heterogeneous IncF group was the predominant replicon type detected in this study. Within the IncF group, we detected the subgroups FIA, FIB, FIC, FIIA, and Frep, with IncFIC and Frep being the most prevalent subgroups. The IncFI plasmid group found in 10 *Salmonella* isolates (Table 3.1) was determined to consist of small plasmids (less than 40 kb in size each). However, the plasmids identified in our study were represented by more than one replicon family in each isolate.

3.3.2 Antimicrobial resistance phenotypes

To determine the AMR phenotypes and MICs for all 14 nalidixic acid-resistant (NAL^r) *E. coli* confirmed transconjugants and the 14 AMR *Salmonella* donor isolates from the environmental source, we conducted antimicrobial susceptibility testing using broth microdilution. The results of transconjugant AST correlated with the AMR profiles and the MICs for the *Salmonella* donor isolates, confirming the successful transfer of plasmids from the donors to the recipient strains (Table 3.2). NAL^r was detected in all 14 transconjugants since the NAL^r *E. coli* JM109 strain was used as a recipient for plasmid transfer. Five out of 14 plasmids were considered multidrug resistant (MDR; resistant to more than three classes of antimicrobials) including plasmids pS6 (*S.* Worthington donor), pS9 and pS10 (*S.* Johannesburg donor), pS24 (*S. enterica* 4,12:i:- donor), and pS27 (*S.* Typhimurium var5- donor) (Table 3.1). The plasmid pS6 showed resistance to sulfisoxazole (FIS), gentamicin (GEN), streptomycin (STR), and tetracycline (TET), while plasmids pS7 and pS8 had the MDR pattern FIS-STR-TET. These three transconjugants were successfully transferred to the recipient *E. coli* from *S.* Worthington donors recovered from NCF1, but only transconjugant

pS6 had a 100% AMR profile that matched that of the donor isolate. Two plasmids, pS9 and pS10, were isolated from transconjugants of *S*. Johannesburg on NCF3 representing identical MDR patterns, with resistance to ampicillin (AMP), amoxicillin-clavulanic acid (AUG2), ceftriaxone (AXO), and cefoxitin (FOX). However, the ceftiofur (XNL) resistance represented in *S*. Johannesburg isolates was not detected in the transconjugants (Table 3.2). The plasmid pS27 from *S*. Typhimurium var5- recovered from NCF5 showed resistance to AMP, chloramphenicol (CHL), FIS, STR, and TET. Plasmids pS12, pS13, pS14, and pS15 isolated from transconjugants of *S*. Typhimurium var5- on farm 3 had the resistance pattern AMP-FIS. The plasmid from NCF6, pS24 with the MDR pattern AMP-FIS-STR-TET, was isolated from an *S. enterica* 4,12:i:- transconjugant. Another plasmid from farm 6 (pS20) from *S*. Rissen was resistant to only TET. All the transconjugants with AMP resistance were selected on Luria-Bertani (LB) plates with AMP and NAL as the markers, while the rest of the transconjugants were selected on NAL and TET marker LB plates.

3.3.3 Determination of antimicrobial resistance genes

Following the conjugation experiment and AST, 14 AMR-encoding genes were tested using a PCR-based method (Table 3.3). Only eight of these marker genes, including *bla*CMY-2, *bla*TEM, *sul*1, *sul*2, *aad*A, *aad*A2*, tet*(A), and *tet*(B), were detected in plasmids. The *bla*CMY-2 gene was detected in a 100-kb IncI1 plasmid (pS9). The *bla*TEM gene was found in an IncFII plasmid (pS27). We detected *tet*(A) or *tet*(B) in plasmids that encoded tetracycline resistance. In plasmids carrying streptomycin resistance, *aad*A1, and *aad*A2 were found. The *sul*1 gene was the most prevalent among plasmids which were resistant to the antimicrobial sulfisoxazole. Plasmids pS14 and pS15 did not test positive for any AMR

genes which were tested in this study. The resistance genotypes of all 14 plasmids are tabulated in Table 3.1.

3.3.4 Plasmid sequencing and analysis

The incompatibility (Inc) group and resistance genes of plasmids were confirmed using sequencing (Table 3.1). Plasmid sequencing was able to identify the replicon families of each individual plasmid. A blastn comparison revealed that 95-kb IncF plasmids from different farms and serotypes (pS9, pS10, pS12, pS13, pS14, pS15, and pS27) (Table 3.1) were identical to another fully sequenced plasmid, pSTY1-H2662 previously isolated from *S.* Typhimurium from human stool (GenBank accession number CP014980) (33). A class 1 integron was identified in plasmids pS6 to pS8 isolated from *S.* Worthington using *in silico* analysis. This integron was comprised of a 5" conserved segment (CS), variable part, and 3" conserved segment (Fig. 3.1, pS7). The unusual variable part contained an ANT(2")-Ia-*aad*A2 gene cassette, which is responsible for aminoglycoside resistance, while the *sul*1 gene was always found in the 3"CS responsible for sulfonamide resistance. In addition, plasmid sequence analysis revealed the presence of VirB-family type IV secretion systems (T4SS)in all 14 plasmids, together with multiple *tra* genes, including *tra*C, *tra*F, *tra*G, *tra*I, *tra*J, *tra*O, and *tra*U. The evolutionary tree of 14 plasmid sequences was created using Geneious R10 software (Fig. 3.2; Appendix 6-7). At 70% similarity, the plasmids from the same *Salmonella* donor were clustered together, including pS6, pS7, and pS8 (from *S.* Worthington) and pS28 and pS29 (from *S. enterica* serotype Ohio). The plasmids with distinct sizes, the 100-kb pS9 and 90-kb pS20, were separated from the other group. Plasmid pS24 was not included in the analysis because of the incomplete sequencing output. The plasmid multilocus sequence

typing (pMLST) database revealed that three 50-kb IncN plasmids isolated from *S.* Worthington belonged to sequence type 5 (ST5). The IncI1 plasmid (pS9) isolated from *S.* Johannesburg was assigned to ST12 and clonal complex 12 (CC-12); another IncI1 plasmid (pS20) isolated from *S.* Rissen was typed as ST155, but the clonal complex was not defined (Table 3.1).

3.4 Discussion

The aim of the study was to characterize the plasmids identified in different AMR *Salmonella* serotypes isolated from a commercial swine farm environment after land application of manure. We also wanted to determine the role of plasmids in the dissemination of AMR genes to other potential bacterial recipients in the environment. The results potentially addressed the key role played by plasmids in the horizontal gene transfer that leads to the rapid proliferation of AMR genes in the environment. It is important to stress that our study was conducted at commercial swine farms and not at a research station in North Carolina, which is one of the top two leading pork-producing states in the United States. The *Salmonella* serotypes carrying multiple plasmids are common in the *Enterobacteriaceae* family (34). However, we focused on large (defined as being $\geq$40 kb in size) plasmids which are abundant in *E. coli* and *Salmonella* and comprise important pools of adaptive and transferable genetic information, especially AMR-corresponding genes, in these bacteria (34, 35). The large plasmids, in the range of 40 to 200 kb, have been suggested to be the necessary markers for extended-spectrum β-lactamases (ESBL), β-lactamase-encoding genes, and plasmid-mediated quinolone resistance (PMQR) (14, 16, 36). In our study, 5 out of 14 plasmids that we detected were 95 kb in size and were isolated from the *S.* Typhimurium

var5- serotype (*n*=5). The plasmid profiles of these five isolates were similar although they were recovered from different farms and at different time points, indicating the persistence of this plasmid in this serotype in the environment after manure deposition. The results correlated with those of a previous study that reported that *Salmonella* plasmids were conserved and primarily serotype specific, including those of *S.* Typhimurium and *S. enterica* serotype Heidelberg, and that they tended to persist for a long period in the environment (34). These plasmids were in contrast to *E. coli* plasmids which were more variable and not specific to particular strains (22, 34). The pS24 plasmid isolated from *S. enterica* 4,12:i:- had a profile similar to that of *S.* Typhimurium plasmids, and the parent strain was also isolated from a different swine farm environment. During the last decade, *S. enterica* 1,4,12:i:-, 1,4,[5],12:i:-, and 4,12:i;- have emerged around the world and have frequently been isolated from human, animal, agricultural production, and environmental sources (37–39). These serotypes are believed to be a mosaic variant of *S.* Typhimurium and are related to plasmid-mediated colistin resistance encoded by the *mcr*-1 gene (37, 39, 81). We detected one *S.* Rissen plasmid of approximately 90 kb that carried a tetracycline resistance marker. This is in comparison to our previous report where we identified from a farm environment in North Carolina a 90- to 100-kb plasmid in a tetracycline resistant *S.* Rissen isolate carrying the *tet*(A) gene (31). This serotype is not common in the U.S. agricultural system and was identified for the first time in North Carolina swine farms in 2009 (42).

Typing of plasmid incompatibility (Inc), the inability of two plasmids of the same family to coexist in the same host cell, classifies plasmids based on their stability during conjugation (43, 82). This classification helps to categorize plasmids into clusters and relies

on their phylogenetic relatedness, distribution in the host cells and environment, and their evolutionary origin (43, 44). Currently, 27 Inc groups are identified among the *Enterobacteriaceae* family (43, 45). On the basis of the PCR-based replicon typing (PBRT) method, 18 Inc groups were detected in our study. We used total plasmid DNA from each isolate in conducting PBRT, so the results did not differentiate individual plasmids in multiplasmid isolates. Most of the isolates were positive for more than one replicon family either because the isolates contained multiple plasmids from different incompatibility groups or because a single plasmid carried replication or partitioning genes from more than one incompatibility group. However, we were able to identify the exact replicon families after assessing the plasmid sequencing data (Table 3.1). We did not differentiate the heterogeneous IncF plasmids into individual groups because of their partitioning of replication genes (34), and the small (<40 kb in size) plasmids were not characterized in this study.

Particular plasmid Inc families, including IncN, IncI1, and IncF, are more frequently associated with the dissemination of AMR genes (14). These three plasmid Inc families have been associated with specific *Salmonella* serotypes and geographic farm areas in our study (34). The IncN family was detected in *S.* Worthington, which was consistently isolated from NCF1, while IncI1 was detected in *S*. Johannesburg isolated from NCF3. Both families are associated with large plasmids related to MDR phenotypes (Table 3.1). The IncF family was detected in multiple serotypes and farms (NCF3, -5, and -6). These findings are in accordance with those of previous studies that found that IncF and IncI1 are the most prevalent replicon types distributed among the *Enterobacteraceae* (14, 34). The IncI and IncF

plasmids generally recovered from *E. coli* and *Salmonella* from human and animal sources are considered the source of several ESBL genes (14, 20, 23).

The IncFI group including FIA, FIB, and FIC, together with the IncFIIA subtype, was the most frequently detected replicon type in this study. All 14 *Salmonella* isolates carried at least one IncF plasmid. Our result supports the view that the IncF (both FI and FII) family was well adapted and commonly distributed in *E. coli* and *Salmonella* (14, 15, 34, 46). Wang et al. (14) reported that IncFIIA was detected only in *S. enterica* serotype Typhimurium, which correlates with our findings; however, we also detected the FIIA type in the *S. enterica* serotype 4,12:i:-. IncF family plasmids have been reported to contribute to the spread of AMR in *Enterobacteriaceae* and have been associated with specific genes conferring resistance to aminoglycosides, β-lactams, and quinolones (43, 46, 47).

Conjugative plasmids of the IncI1 replicon type were usually associated with multiple resistance compounds, especially extended-spectrum cephalosporinases of both the CTX-M and CMY types (47–49). The IncI1 plasmids carrying TEM-52 have been identified in *E. coli* and *Salmonella* cultured from humans and from chicken and turkey products in the European Union (50–52). The *bla*CMY-IncI1 plasmids linked to poultry, ground beef, and tomato sources have been identified to be responsible for ceftriaxone-resistant *Salmonella* outbreaks in the United States during 2011 and 2012 (18). Reports indicated that *Salmonella enterica* serotypes Heidelberg, Infantis, Typhimurium, and Newport were associated with IncI plasmids carrying the *bla*CMY gene. Similar to results of our study, IncI plasmids carrying the *bla*CMY gene were identified in a ceftriaxone-resistant *S. enterica* serotype Johannesburg isolate from a commercial swine farm environment sampled in our study.

IncN plasmids are the major vehicles for the dissemination of PMQR and ESBL genes, including *bla*CTX-M (22, 53, 54). In contrast to results of our study, IncN plasmids were identified in *S*. Worthington transconjugants and exhibited resistance to sulfisoxazole, streptomycin, and tetracycline but not to quinolones and ampicillin. Thus, characterization based on plasmid profiling and the corresponding Inc group using the PBRT technique is an essential tool for plasmid epidemiological surveillance, enhancing discrimination between *Salmonella* serotypes and tracing the spread of AMR genes (14, 16).

Multiple MDR-coding genes were found in plasmids. We detected plasmids carrying *sul*1 and *sul*2 genes conferring sulfisoxazole resistance, while plasmids with streptomycin resistance carried the *aad*A and *aad*A2 genes. Similarly, the *tet*(A) and *tet*(B) genes were found in plasmids in *Salmonella* strains that were resistant to tetracycline. β-Lactamase-encoding (*bla*) genes, including *bla*TEM and *bla*CMY, were detected in the plasmids which encoded the resistance to ampicillin and cephalosporin group antimicrobials. Several mechanisms are available for *bla* genes to support HGT between bacteria, thereby ensuring the spread of these markers to new hosts and the environment (14, 55). The heavy use of specific antimicrobials such as tetracycline plays a key role in plasmid dissemination and allows for the selection and enrichment of bacteria with multidrug-resistant plasmids (22, 56, 57).

The class 1 integron with an ANT(2”)-Ia-*aad*A2 gene cassette was detected in plasmids pS6 to pS8 retrieved from *S.* Worthington (Fig. 3.1, pS7). The integron had an unusual organization, with an ANT(2”)-Ia gene cassette which is responsible for resistance against gentamicin (58). The gentamicin resistance was not identified in pS7 but in

Salmonella isolate S7 (pS7 donor) and pS6 (Tables 3.1 and 3.2). After BLAST analysis at NCBI, pS6 to pS8 showed genetic relatedness to a *Klebsiella pneumoniae* MDR IncN plasmid reported from Japan (59). However, the *K. pneumoniae* plasmid harbored different resistance genes than those we detected in the *Salmonella* serotypes from our study. The integrons are able to locate on either a chromosome or a mobile genetic element such as a plasmid (60). Several studies have stated that the integrons harboring *aad*A or a variant of *aad*A genes are common among *Salmonella* species (10, 61–63). The variable parts of integrons might be composed of variants of *aad*, *dfr*, or *bla* genes that contribute to aminoglycoside, sulfonamide, and cephalosporin resistance, respectively (10, 61). *S. enterica* serotype Worthington detected in our study is commonly found in poultry, poultry products, and the environment in several parts of the world and harbors integrons either on the chromosome or plasmids (62, 64–66). The presence of genetic elements such as integrons, transposons, and plasmids has consequently been associated with multidrug resistance phenotypes among *Salmonella* isolates (10). Our study reports an emerging multidrug-resistant clone isolated from *Salmonella* serotypes in a commercial swine farm environment carrying a large conjugative plasmid with an ANT(2")-Ia-*aad*A2 gene cassette located on an integron.

Though the *Salmonella* plasmids were transferred to an *E. coli* JM109 recipient under laboratory conditions, the presence of VirB-family type IV secretion systems (T4SS) and *tra* genes in our study confirms that HGT by conjugation is likely to occur in the environment. The T4SS in gram-negative bacteria functionally encompass the conjugation system and the effector translocators for interbacterial transfer of AMR genes, virulence determinants, and

genes encoding other traits beneficial to the host (67). IncN plasmids (pS6 to pS8) and IncI1 plasmids (pS9 [100-kb] and pS20) employed *tra*J, which they has ability to conjugate, and the conjugation process could be stimulated approximately 100-fold, demonstrating functional conservation of a significant regulatory feature of F-like conjugation modules (68).

The phylogenetic tree of 14 plasmids (Fig. 3.2; Appendix 5.6; Appendix 5.7) at 70% similarity suggested that the plasmids analyzed in our study were clustered based on the *Salmonella* donor serotypes, such as the *S*. Worthington cluster (pS6 to pS8) and S. Ohio cluster (pS28 and pS29). Within three Inc groups (IncI1, IncN, and IncF), the phylogenetic analysis also suggested the existence of an Inc group that is serotype specific (34). Based on the pMLST database, all IncN (pS6 to pS8) plasmids which were specific to *S. enterica* serotype Worthington belonged to the same ST5. These results were in accordance with the BLAST output for individual plasmids and the *Salmonella* clustering done by pulsed-field gel electrophoresis (PFGE) in our previous study (13).

Our study demonstrated that identical plasmids were recovered from different *Salmonella* serotypes isolated either from the same or different farm environments. Our findings provide evidence of a single, large 95-kb IncF plasmid being distributed across the swine production systems in North Carolina among different serotypes of *Salmonella*. In addition, we found that AMR plasmids were able to persist in the swine farm environment after manure application for a minimum period of 21 days (final sampling time point). The AMR determinants on these plasmids were transferable among *Salmonella* serotypes, which underlined the fact that manure deposition enriches the environmental resistome. We

recommend conducting longitudinal studies on commercial food animal farms to determine the role of manure deposition on the environmental dissemination of AMR genes.

3.5 Materials and methods

3.5.1 *Salmonella* serotype selection

A total of 168 AMR *Salmonella* isolates from commercial swine farm environments in North Carolina during 2013 to 2015 were tested for their plasmid components. The details of farm distribution, waste management systems, sample collection, and *Salmonella* isolation were described in a previous study (13). Briefly, manure samples from a lagoon and soil samples before and after manure spray application were collected on the first day (day 0) of the farm visit. The subsequent soil samples were collected on day 7, day 14, and day 21 from the same plots as on day 0. The serotyping, antimicrobial susceptibility testing (AST), and pulsed-field gel electrophoresis (PFGE) were performed for phenotypic and genotypic characterization of the *Salmonella* strains. The *Salmonella* isolates selected for plasmid characterization were chosen based on their temporal and spatial relationships, AMR profiles, AMR determinants, and PFGE fingerprint profiles. Based on the above criteria, a total of 14 isolates were finally selected for plasmid analysis and sequencing (Table 3.1). All isolates were maintained at -80°C in brucella broth (Difco, Becton-Dickinson, USA) until further characterization.

3.5.2 Conjugation experiments

Conjugation experiments were conducted to evaluate intra- and interserovar transmission of AMR genes among AMR *Salmonella* serotypes. Fourteen AMR *Salmonella* isolates were selected to serve as donor strains, and the nalidixic acid-resistant (NAL^r)

Escherichia coli JM109 strain was used as a recipient strain. A heat shock assay modified from Zeng et al. (69) was utilized for performing conjugation experiments. In brief, a loopful of overnight culture of the donor strain was gently mixed in Luria-Bertani (LB) broth (Difco, Becton-Dickinson, USA) with *E. coli* JM109. The donor and recipient DNA mixtures were kept on ice for 20 to 30 min, given heat shock in a water bath at 42°C for 30 to 60 s, and moved back on ice for 2 min. We added 250 to 1,000 µl of LB broth and incubated the culture mix at 37°C in a shaking incubator for 45 to 60 min. The culture mixtures were transferred to selective LB plates (Criterion; Hardy Diagnostics, USA) containing nalidixic acid (50 µg/ml) and one of the antimicrobials, depending on the resistance profile of the donor strain, and incubated at 37°C overnight. Transconjugants were confirmed on nontyphoidal *Salmonella* chromogenic plates (CHROMagar, Paris, France) and xylose lactose tergitol (XLT4) agar plates (Criterion; Hardy Diagnostics, USA). The antimicrobials and the concentrations used are as follows: ampicillin, 100 µg/ml; nalidixic acid, 50 µg/ml; and tetracycline, 20 µg/ml.

3.5.3 Antimicrobial susceptibility testing

The transconjugant AMR and MIC profiles were determined by the broth microdilution method using a Gram-negative Sensititre® (CMV3AGNF) plate (Trek Diagnostic Systems, OH). The panel of 14 antimicrobials tested include amoxicillin-clavulanic acid (AUG2; 1/0.5 to 32/16 µg/ml), ampicillin (AMP; 1 to 32 µg/ml), azithromycin (AZI; 0.12 to 16 µg/ml), cefoxitin (FOX; 0.5 to 32 µg/ml), ceftiofur (XNL; 0.12 to 8 µg/ml), ceftriaxone (AXO; 0.25 to 64 µg/ml), chloramphenicol (CHL; 2 to 32 µg/ml), ciprofloxacin (CIP; 0.015 to 4 µg/ml), gentamicin (GEN; 0.25 to 16 µg/ml), nalidixic

acid (NAL; 0.5 to 32 µg/ml), streptomycin (STR; 2 to 64 µg/ml), sulfisoxazole (FIS; 16 to 256 µg/ml), trimethoprim-sulfamethoxazole (SXT; 0.12/2.38 to 4/76 µg/ml), and tetracycline (TET; 4 to 32 µg/ml). The MICs were determined, and breakpoints were interpreted based on the Clinical and Laboratory Standards Institute standards (CLSI) for broth microdilution (70, 71) and the National Antimicrobial Resistance Monitoring System (NARMS) (72). *E. coli* ATCC 25922 was used as a quality control strain. The transconjugants with MICs in the intermediate level were categorized as susceptible to avoid overestimation of resistance. The transconjugants with resistance to three or more classes of antimicrobials were classified as multidrug resistant (MDR).

3.5.4 Plasmid isolation

Plasmid DNA was isolated from the confirmed transconjugant (NAL^r *E. coli* JM109) cultures by the modified alkali lysis method described by Sambrook et al. (73), which is suitable for the isolation of both large and small plasmids. The purified DNA concentrations of the plasmid extracts were calculated by measuring the absorbance at 260 and 280 nm using a NanoDrop ND-2000 Spectrophotometer (NanoDrop; Wilmington, DE) and Qubit, version 3.0, fluorometer (Invitrogen, Carlsbad, CA) to ensure that there was adequate plasmid DNA for sequencing. The plasmid DNA was stored frozen at -20°C until required.

3.5.5 PCR amplification of resistance genes

The presence of resistance genes on plasmids of specific AMR *Salmonella* phenotypes was detected using PCR (31, 74). Overall, genes encoding resistance to ampicillin and cephalosporin (*bla*PSE-1, *bla*TEM, and *bla*CMY-2), chloramphenicol (*cml*A), streptomycin (*aad*A1, *aad*A2, *str*A, and *str*B), sulfisoxazole (*sul*1 and *sul*2), and tetracycline

[*tet*(A), *tet*(B), *tet*(C), and *tet*(G)] were tested. Template plasmid DNAs were extracted by the modified alkali lysis method mentioned above. The primers, amplicon sizes, and references used to detect the presence of the selected AMR genes are listed in Table 3.3. The PCR conditions for all resistance genes, except the *cml*A and *sul*1 genes, included an initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 54°C, extension for 1 min at 72°C, and a final extension at 72°C for 7 min. For the *cml*A and *sul*1 genes, the PCR conditions used have been described previously (75). Briefly, an initial denaturation at 94°C for 5 min was followed by 30 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 57°C, extension for 1 min at 72°C, and a final extension at 72°C for 5 min. *Salmonella enterica* isolates carrying resistance genes and characterized in earlier studies were used as positive controls (31).

3.5.6 Plasmid PCR-based replicon typing (PBRT)

Single and multiplex PCRs were run to identify different incompatibility (Inc) groups, including FIA, FIB, FIC, HI1, HI2, I1-Iɣ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA. The primers and PCR running conditions have been described in a previous study (45). The purified plasmid DNA from the modified alkali lysis method was used as the template DNA. PCR running conditions used for the five multiplex PCRs and three single PCRs included an initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 30 s at 60°C, and elongation for 1 min at 72°C, with a final extension of 5 min at 72°C. The single PCRs for Frep were performed under the same amplification conditions but with an annealing temperature of 52°C. The PCR products were

electrophoresed on a 1.5% agarose gel in Tris-acetate-EDTA (TAE) buffer and UV visualized by staining with ethidium bromide.

3.5.7 Plasmid sequencing, assembly, and annotation

Isolated plasmid DNA libraries were prepared for sequencing using a Nextera XT kit (Illumina, San Diego, CA). Multiplexed sequencing of these libraries was done with a single run on an Illumina MiSeq using 2-by-250- or 2-by-300-bp paired-end reads (MiSeq reagent kit, version 3). Following demultiplexing, sequences were analyzed using CLC Genomic Workbench 10 (Qiagen, Valencia, CA). For analyzing plasmid content, *de novo* assembly of unused reads into new contigs was applied. The initially assembled contigs were analyzed using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (BLAST). In addition, individual sequence reads were mapped back to the assembled plasmids to confirm that there were continuous overlapping reads over the entire length of the assembled plasmid. Following completion of plasmid assembly, the plasmid sequences were run through a BLAST search individually and compared to GenBank sequences. The open reading frame (ORF) of each gene in plasmid contigs was identified, and the particular genes of interest were annotated using Geneious R10 software (BioMatters, New Zealand). Manual trimming and editing of terminally redundant contig ends generated circular plasmid genomes. The complete plasmid sequences were visualized using plasmid mapping in the CLC Workbench and deposited in the GenBank under prospective accession numbers.

3.5.8 Comparative genotypic analysis

To further characterize the plasmids and compare their profiles, we mapped the PCR primers described by Carattoli et al. (45) to the assembled plasmid sequences with a BLAST

search configured for short reads. Based on the annotations and BLAST output, the plasmids were assessed for the presence of known AMR genes, plasmid transfer (*tra*) genes, and mobile genetic elements, including class I integrons and transposons. The assembled plasmid sequences submitted to a BLAST search were compared to previously sequenced plasmids in GenBank. We identified 14 plasmid sequences and analyzed them for variation using the Geneious R10 software (BioMatters, New Zealand) global alignment with 70% similarity to construct neighbor-joining trees using the Tamura-Nei genetic distance model. In addition, all 14 plasmid sequences were typed by pMLST as previously described (41) and assigned to STs according to the plasmid MLST database (https://pubmlst.org/plasmid/) for ST prevalence analysis.

3.5.9 Accession number(s)

The sequencing output of the 14 *Salmonella* plasmids was submitted to the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA293224. Individual plasmid sequence reads have been deposited in the Sequence Read Archive (SRA) as BioSample numbers SAMN07345795 to SAMN07345807.

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Table 3.1 Conjugative resistance plasmid content of 14 environmental isolates harboring AMR genes recovered from *Salmonella* donor isolates after manure application on commercial swine farms in North Carolina

***Salmonella* donor isolate**			**Plasmid**					
Farm[a] and source	**Day of sampling**	**Serotype**	**ID**	**Size (kb)**	**Inc group[b]**	**pMLST[c]**	**R-pattern[d] (MIC; µg/ml)**	**AMR gene**
NCF1								
Lagoon	0	Worthington	pS6	50	N(50k), FI	ST5 (N)	FIS(>256), GEN(16), STR(64), TET(>32)	*sul*1, *aadA*2, *tet*(A)
Soil	0	Worthington	pS7	50	N(50k), FI	ST5 (N)	FIS(>256), STR(64), TET(>32)	*sul*1, *aadA*2, *tet*(A)
Soil	7	Worthington	pS8	50	N(50k), FI	ST5 (N)	FIS(>256), STR(64), TET(>32)	*sul*1, *aad*A2, *tet*(A)
NCF3								
Lagoon	0	Johannesburg	pS9	100, 95	I1(100k), FI(95k)	ST12 (I1)	AMP(>32), AUG2(32/16), AXO(16), FOX(32)	bla_{CMY-2}
Soil	7	Johannesburg	pS10	95	I1, FI(95k)	-	AMP(>32), AUG2(32/16), AXO(16), FOX(32)	bla_{CMY-2}
Lagoon	0	Typhimurium var5-	pS12	95	FII, FI	-	AMP(>32), FIS(>256)	*sul*1
Soil	7	Typhimurium var5-	pS13	95	FII, FI	-	AMP(>32), FIS(>256)	*sul*1
Soil	14	Typhimurium var5-	pS14	95	FII	-	AMP(>32), FIS(>256)	-
Soil	21	Typhimurium var5-	pS15	95	FII	-	AMP(>32), FIS(>256)	-
NCF6								
Lagoon	0	Rissen	pS20	90	I1(90k), FI	ST155 (I1)	TET(>32)	*tet*(A), *tet*(B)
Soil	0	4,12:i:-	pS24	95	FII, FI	-	AMP(>32), FIS(>256), STR(>64), TET(>32)	bla_{TEM}, *sul*2, *aad*A

Table 3.1 Continued

***Salmonella* donor isolate**			**Plasmid**					
Farm[a] and source	**Day of sampling**	**Serotype**	**ID**	**Size (kb)**	**Inc group[b]**	**pMLST[c]**	**R-pattern[d] (MIC; µg/ml)**	**AMR gene**
NCF5								
Lagoon	0	Typhimurium var5-	pS27	95	FII, FI	-	AMP(>32), CHL(>32), FIS(>256), STR(32), TET(32)	*sul*1, *aad*A2
Lagoon	0	Ohio	pS28	40	FI	-	TET(>32)	*tet*(A)
Soil	0	Ohio	pS29	40	FI	-	TET(>32)	*tet*(A)

[a] NCF, North Carolina farm.

[b] Incompatibility group based on PBRT (45).

[c] Sequence type (ST) based on pMLST (https://pubmlst.org/plasmid/) (41).

[d] Nalidixic acid (NAL) resistance was not detected in the plasmid isolated from the transconjugant. MIC ranges of the drugs are as follows: amoxicillin-clavulanic acid (AUG2), 1/0.5 to 32/16 µg/ml (breakpoint, ≥32/16 µg/ml); ampicillin (AMP), 1 to 32 µg/ml (breakpoint, ≥32 µg/ml); cefoxitin (FOX), 0.5 to 32 µg/ml (breakpoint, ≥32 µg/ml); ceftriaxone (AXO), 0.25 to 64 µg/ml (breakpoint, ≥4 µg/ml); chloramphenicol (CHL), 2 to 32 µg/ml (breakpoint, ≥32 µg/ml); gentamicin (GEN), 0.25 to 16 µg/ml (breakpoint, ≥16 µg/ml); streptomycin (STR), 32 to 64 µg/ml (breakpoint, ≥32 µg/ml); sulfisoxazole (FIS), 16 to 256 µg/ml (breakpoint, ≥512 µg/ml); and tetracycline (TET) 4 to 32 µg/ml (breakpoint, ≥16 µg/ml)

Table 3.2 Antimicrobial susceptibilities with MICs of AMR environmental *Salmonella* isolates and corresponding *E. coli* transconjugants

Salmonella isolate or transconjugant[a]	MIC[b] (µg/ml)													
	AMP	AUG2	AXO	AZI	CHL	CIP	FIS	FOX	GEN	NAL	STR	SXT	XNL	TET
S6	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**64**	<0.12/2.38	1	**>32**
TC-S6	<1	2/1	<0.25	2	8	0.06	**>256**	4	**16**	**>32**	**64**	<0.12/2.38	0.5	**>32**
S7	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**16**	2	**>64**	<0.12/2.38	0.5	**>32**
TC-S7	2	2/1	<0.25	2	8	0.12	**>256**	4	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S8	<1	<1/0.5	<0.25	4	8	<0.015	**>256**	4	**>16**	2	**64**	<0.12/2.38	0.5	**>32**
TC-S8	2	2/1	<0.25	2	8	0.12	**>256**	2	8	**>32**	**64**	<0.12/2.38	0.5	**>32**
S9	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	4	<0.12/2.38	**>8**	<4
TC-S9	**>32**	**32/16**	**16**	2	8	0.12	<16	**32**	<0.25	**>32**	4	<0.12/2.38	4	<4
S10	**>32**	**32/16**	**16**	8	8	0.03	256	**32**	0.5	4	8	<0.12/2.38	**>8**	<4
TC-S10	**>32**	**32/16**	**16**	2	8	0.25	<16	**>32**	0.5	**>32**	4	<0.12/2.38	4	<4
S12	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	0.5	<4
TC-S12	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S13	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	0.25/4.75	0.5	<4
TC-S13	**>32**	8/4	<0.25	4	8	0.12	**>256**	4	0.5	**>32**	8	<0.12/2.38	1	<4
S14	**>32**	8/4	<0.25	4	8	<0.015	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S14	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	8	<0.12/2.38	1	<4
S15	**>32**	<1/0.5	<0.25	4	8	0.25	**>256**	2	0.5	4	8	<0.12/2.38	1	<4
TC-S15	**>32**	8/4	<0.25	4	8	0.25	**>256**	2	0.5	**>32**	16	0.25/4.75	1	<4
S20	<1	<1/0.5	<0.25	8	8	0.03	64	4	0.5	4	4	<0.12/2.38	1	**>32**
TC-S20	2	2/1	<0.25	4	8	0.12	<16	4	<0.25	**>32**	4	<0.12/2.38	<0.12	**>32**
S24	**>32**	4/2	<0.25	8	8	0.03	**>256**	2	0.5	8	**>64**	<0.12/2.38	1	**>32**
TC-S24	**>32**	8/4	<0.25	4	8	0.12	**>256**	2	0.5	**>32**	**>64**	<0.12/2.38	0.5	**>32**
S27	**>32**	**32/16**	**8**	8	**>32**	<0.015	**>256**	16	0.5	4	**>64**	<0.12/2.38	**8**	**>32**
TC-S27	**>32**	8/4	<0.25	4	**>32**	0.12	**>256**	2	<0.25	**>32**	**32**	<0.12/2.38	0.5	**32**
S28	<1	<1/0.5	<0.25	8	8	<0.015	64	2	<0.25	2	4	<0.12/2.38	1	**>32**
TC-S28	2	2/1	<0.25	8	8	0.12	<16	8	<0.25	**>32**	4	<0.12/2.38	0.5	**>32**
S29	<1	<1/0.5	<0.25	4	8	<0.015	64	2	0.5	4	8	<0.12/2.38	1	**>32**
TC-S29	2	2/1	<0.25	4	8	0.12	<16	2	<0.25	**>32**	<2	<0.12/2.38	0.5	**>32**

Table 3.2 Continued

[a] *E. coli* transconjugants are indicated by designations beginning with "TC." *Salmonella* isolate designations begin with the letter "S."

[b] MIC ranges of the drugs are as follows: amoxicillin/clavulanic acid (AUG2; 1/0.5-32/16 μg/ml; breakpoint≥32/16), ampicilin (AMP; 1-32 μg/ml; breakpoint≥32), azithromycin (AZI; 0.12-16 μg/ml; breakpoint≥32), cefoxitin (FOX; 0.5-32 μg/ml; breakpoint≥32), ceftiofur (XNL; 0.12-8 μg/ml; breakpoint≥8), ceftriaxone (AXO; 0.25-64 μg/ml; breakpoint≥4), chloramphenicol (CHL; 2-32 μg/ml; breakpoint≥32), ciprofloxacin (CIP; 0.015-4 μg/ml; breakpoint≥4), gentamicin (GEN; 0.25-16 μg/ml; breakpoint≥16), nalidixic acid (NAL; 0.5-32 μg/ml; breakpoint≥32), streptomycin (STR; 32-64 μg/ml; breakpoint≥32), sulfisoxazole (FIS; 16-256 μg/ml; breakpoint≥512), trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 μg/ml; breakpoint≥4/76), and tetracycline (TET; 4-32 μg/ml; breakpoint≥16).

Boldface indicates resistance of the *Salmonella* isolate or transconjugant to the antimicrobial.

Table 3.3 Primers used for PCR detection of resistance genes

Genes	Forward oligonucleotide sequence (5'to 3')	Reverse oligonucleotide sequence (5' to 3')	Expected Size (bp)	Ref.
bla$_{CMY-2}$	GACAGCCTCTTTCTCCACA	TGGAACGAAGGCTACGTA	1015	39
bla$_{PSE-1}$	TTTGGTTCCGCGCTATCTG	TACTCCGAGCACCAAATCCG	150	40
bla$_{TEM}$	GCACGAGTGGGTTACATCGA	GGTCCTCCGATCGTTGTCAG	860	41
*aad*A	GTGGATGGCGGCCTGAAGCC	AATGCCCAGTCGGCAGCG	528	42
*aad*A2	CGGTGACCATCGAAATTTCG	CTATAGCGCGGAGCGTCTCGC	250	43
*str*A	CCTGGTGATAACGGCAATTC	CCAATCGCAGATAGAAGGC	548	42
*str*B	ATCGTCAAGGGATTGAAACC	GGATCGTAGAACATATTGGC	509	42
*sul*1	CGGACGCGAGGCCTGTATC	GGGTGCGGACGTAGTCAGC	591	44
*sul*2	GCGCTCAAGGCAGATGGCATT	GCGTTTGATACCGGCACCCGT	285	41
*cml*A	TGGACCGCTATCGGACCG	CGCAAGACACTTGGGCTGC	641	44
tet(A)	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	210	45
tet(B)	TTGGTTAGGGGCAAGTTTTG	GTAATGGGCCAATAACACCG	659	45
tet(C)	CTTGAGAGCCTTCAACCCAG	ATGGTCGTCATCTACCTGCC	418	45
tet(G)	CAGCTTTCGGATTCTTACGG	GATTGGTGAGGCTCGTTAGC	844	45

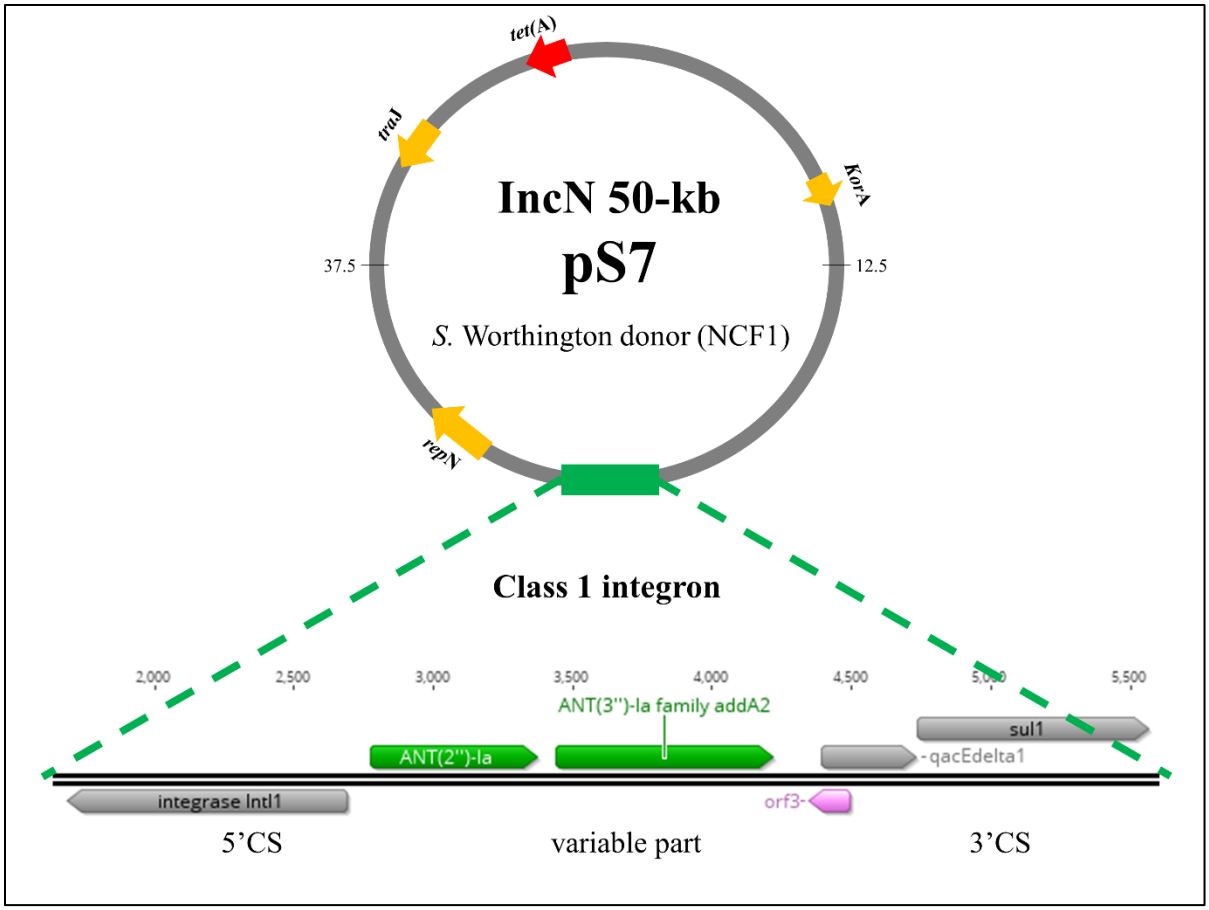

Figure 3.1 Schematic representation of a class 1 integron in 50-kb IncN plasmid pS7: in the 5”conserved segment, the *intI*1 integrase gene; in the variable region, ANT(2”)-Ia, producing the aminoglycoside resistance enzyme, and *aad*A2, an ANT(3”)-Ia family *aad*A2 gene producing streptomycin resistance; in the 3”conserved segment, *qacE*Δ1, a partially deleted gene that encodes quaternary ammonium compound resistance, *sul*1, producing sulfonamide resistance, and *orf*3, of unknown function, on the gene cassette recognized by the integrase. Arrows indicate the direction of the coding sequence.

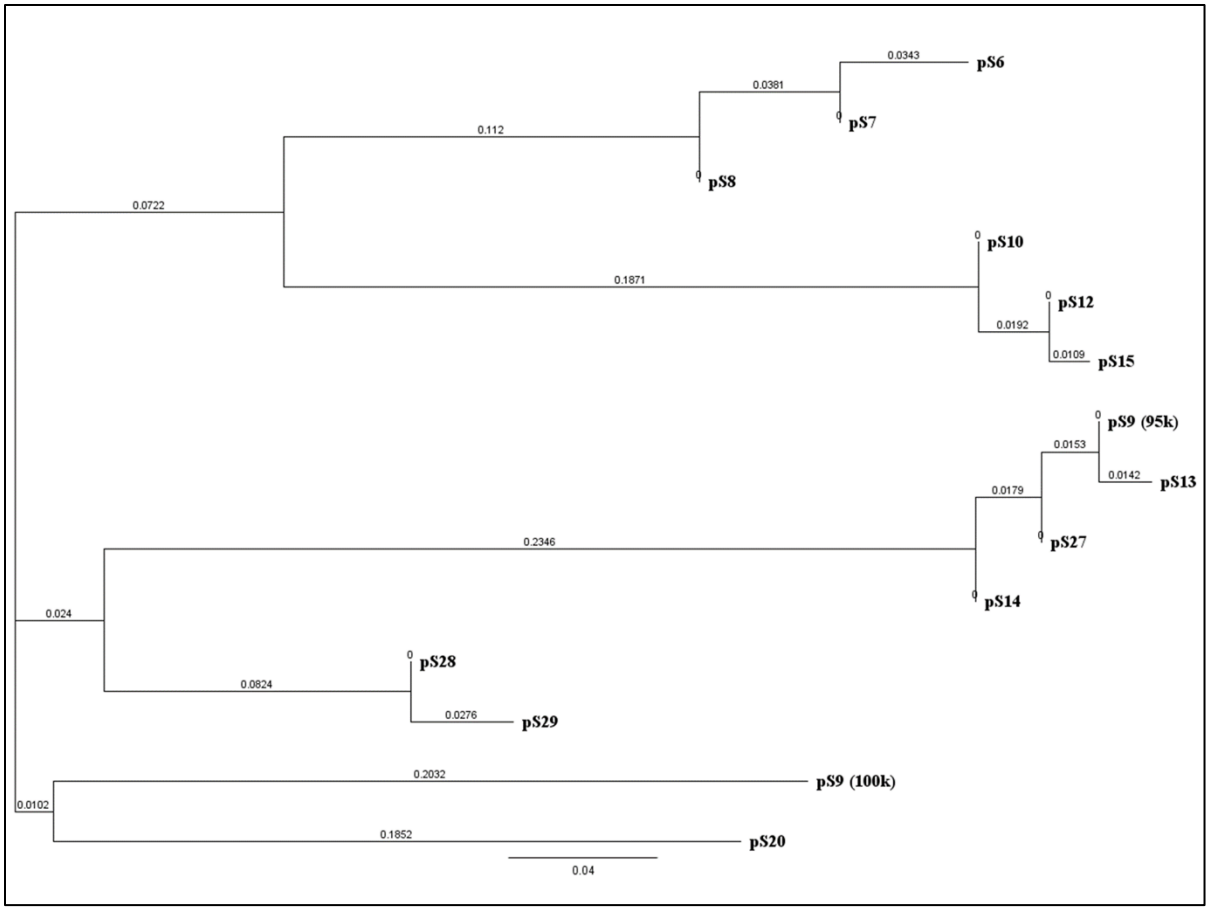

Figure 3.2 Phylogenetic diversity for sequences of 14 plasmids acquired from environmental *Salmonella* isolates. Evolutionary distances between plasmids were computed using a neighbor-joining algorithm. The distance was obtained from pairwise alignments with 70% similarity and no outgroup. The plasmid label names relate to data in Table 3.1. Phylogenetic analyses were conducted in Geneious R10.

4. CHAPTER IV: Whole genome sequencing analysis of multiple *Salmonella* serotypes provides insights into phylogenetic relatedness, antimicrobial resistance, and virulence markers across humans, food animals and environmental sources

Presented here is the manuscript titled "Whole genome sequencing analysis of multiple *Salmonella* serotypes provides insights into phylogenetic relatedness, antimicrobial resistance, and virulence markers across humans, food animals and environmental sources", is submitted the present year (2017) to the Journal of Clinical Microbiology and under the peer reiew. Additional data are presented in the Appendix.

4.1 Abstract

Salmonella enterica is a significant foodborne pathogen, which can be transmitted via several distinct routes, and worryingly displays development of antimicrobial resistance (AMR). In this study, whole genome sequencing (WGS) was applied to better understand the epidemiology of antimicrobial resistant *Salmonella* from clinical cases in humans, food animals and the environment. A total of 200 *Salmonella* isolates recovered from human (*n*=44), swine (*n*=32), poultry (*n*=22), and environment (*n*=102) were used for genome sequencing, and the assembled genomes were used for *in silico* prediction of serotype and the presence of virulence genes in the VFDB database, and phylogenetically clustered using core genome single nucleotide polymorphism (SNP) and feature frequency profiling (FFP). Furthermore, AMR was studied both by genotypic prediction using five curated databases, and compared to phenotypic resistant *Salmonella* using broth microdilution for 15 antimicrobials. Both core genome SNP-based and FFP-based phylogenetic trees showed consistent clustering of isolates into the respective serotypes, and suggested clustering of

isolates based on the source of isolation. The overall correlation of phenotypic and genotypic antimicrobial resistant *Salmonella* was 87.61% and 97.13% for sensitivity and specificity, respectively. AMR and virulence genes clustered with the *Salmonella* serotypes, while there were also associations between the presence of virulence genes in both animal/environmental isolates and human clinical samples. Our study shows that WGS is the useful tool for *Salmonella* phylogenetic analysis, resistance and virulence gene predictions. The isolates from human had a close relationship with animal and environment isolates which are considered to be the sources for *Salmonella* dissemination.

4.2 Introduction

Infection with antimicrobial resistant *Salmonella* in humans and animals is a global threat that has caught the public attention in several countries around the world (1-3). Human foodborne salmonellosis causes an estimated 100,000 domestic cases and 40 deaths annually in the United States (1). The U.S. Department of Health and Human Services reported an increase in *Salmonella* infections from 13.6 to 16.4 cases per 100,000 population, which represented a 17.1% increase from 1997 to 2011 (4). In the European Union, *Salmonella*-infected gastroenteritis was the second most frequently reported foodborne illnesses with 91,408 clinical cases reported by 30 EU/EEA countries, and a confirmed case rate of 25.4 cases per 100,000 population in 2014 compared to 21.4 cases per 100,000 population in 2013 which represented a 19% increase in the notification rate (3).

Antimicrobial use in livestock and the association to resistant *Salmonella* infection in humans has always been of concern to public health agencies. Given the ever-growing requirement to maintain the efficacy of antimicrobials used to treat AMR bacterial foodborne

infection in humans, the antimicrobial use in veterinary practices is being re-evaluated at an extensive scale (5-7). Humans and animals are linked to each other through the environmental reservoirs which have long been implicated as a source of *Salmonella* found in human and animals (6, 8). The antimicrobial use in human, environment and the integrated managements in food animal production create selection pressure on *Salmonella* to develop antimicrobial resistance (AMR) (6, 9). Our previous studies reported the persistence and dissemination of multiple antimicrobial resistant *Salmonella* serotypes along with their determinants in the environment of commercial swine operation due to the manure application on land (10, 11).

Multiple *Salmonella* serotypes, including Agona, Anatum, Derby, Heidelberg, Infantis, Kentucky, Muenchen, Newport, Schwarzengrund, and Typhimurium are commonly detected in food animals, food products, and agricultural environments, and are associated with resistant *Salmonella* infection in human (12-15). The Centers for Disease Control and Prevention (CDC) reported that human *Salmonella* infections caused by monophasic 4,[5],12:i:- which is in the top 4 of the most frequently reported *Salmonella* serotypes, and the incidence of this monophasic variant of Typhimurium continues to rise while the incidence of the other serotypes is decreasing (14). Increase in the frequency of this serotype in human cases is paralleled by a similar increase in swine and environmental detection of this serotype variant recently (10, 11, 16). However, there are gaps that still exist in our understanding of the temporal and spatial connection of resistant *Salmonella* transmission within humans, animals, and the environment sources.

A number of studies have used the classical molecular typing methods such as pulsed-field gel electrophoresis (PFGE), multilocus sequence-based typing (MLST), and multilocus variable-number tandem repeat analysis (MLVA) to assess the relatedness and the subsequent transmission of AMR *Salmonella* in human, animals, and environment (17-19). However, the limitation of these methods lies in insufficient discriminatory power to separate closely related *Salmonella* isolates in outbreak investigations and to differentiate between the intra-serotype isolates from different hosts (18-20). The use of whole genome sequencing (WGS) has had a major impact on the study of the molecular epidemiology of antimicrobial resistant bacterial pathogens associated and transmitted between human, animal and environmental sources. A WGS study in Denmark reported that SNP, pan-genome, k-mer and nucleotide difference trees were superior to the classical typing method and evaluated the association of the isolates to specific outbreaks of *S.* Typhimurium (21). Additionally, the WGS was used to identify known antimicrobial resistant determinants among strains of *Escherichia coli* and *Salmonella* (22, 23). The objective of this study was to use the WGS to analyze multiple AMR *Salmonella* serotypes isolated from human, food animals and environment. In addition, the capability of WGS to predict antimicrobial resistance and virulence genes in AMR *Salmonella* retrieved from different sources was evaluated.

4.3 Results

4.3.1 *Salmonella* serotyping based on WGS

The 200 *Salmonella* sequences in this study selected from human clinical cases, swine, poultry, and environmental samples were serotyped using the *Salmonella In Silico* Typing Resource (SISTR) platform for serotype confirmation (24), and showed a high level

of diversity (Table 4.1). The serotypes were also confirmed using the Kauffman-White scheme. The predominant serotypes from different sources were Derby (*n*=21), Kentucky (*n*=5), Johannesburg (*n*=9), Mbandaka (*n*=12), Rissen (*n*=14), Schwarzengrund (*n*=22), Senftenberg (*n*=12), Typhimurium (*n*=39), and 4,[5],12:i:- (*n*=8). The assembly metrics computed by QUAST v4.5 (25) such as N50, largest contig, number of contigs, total length, L50 were tabulated as shown in Table S1.

4.3.2 Comparison of FFP with SNP-based phylogeny of *Salmonella* isolates

The 200 *Salmonella* genomes were assessed for their phylogenetic relationships using core genome SNPs with the ParSNP program (26) and the alignment-free feature frequency profiling using purine-pyrimidine words (FFPry) (27). Isolates clustered according to serotype with both analysis methods, and the topology of the resulting phylogenetic trees was very similar (Fig. 4.1; Appendix 9). Although the order of specific serotypes did differ. All 200 *Salmonella* genomes clustered into 13 different major groups matching the respective serotypes in both parSNP and FFPry trees. In addition, many major serotype clusters were comprised of the genomes from different sources of origin including human, animal, and the environment. There were also several singleton genomes that did not cluster into any major serotype-associated group. Therefore, these differences have a relatively small effect on the general structure of the trees and the clustering observed.

We focused on the major clusters for serotypes Typhimurium, Derby, Schwarzengrund, and Rissen (Fig. 4.2-4.5; SNP trees, Appendices 14-17; SNP & FFP trees, Appendices 10-13; other clusters). As described previously, these clusters were comprised of the genomes from multiple sources. *S.* Typhimurium and *S.* 4,[5],12:i:- genomes recovered

from human, swine, poultry, and environment clustered together (Fig. 4.2; Appendix 14). The genomes from the same origin have a close relationship as indicated by the positioning on the phylogenetic SNP tree. However, a human clinical fecal (HS71549) was closely grouped along with environmental isolates from the commercial swine farms. Another human case genome (HS5826) was placed near the swine samples on the tree. The genomes of serotype 4,[5],12:i:- recovered from both chicken fecal and environment were grouped close to each other, most likely because they originated from the same farm in Tennessee.

The isolates with serotype Derby showed little variation in the core genome, nor was any specific clustering linked with human, swine, and environmental sources (Fig. 4.3; Appendix 15). In contrast, the isolates of *S.* Schwarzengrund (Fig. 4.4; Appendix 16) showed isolation source-specific clustering of human isolates separate from the group of chicken fecal and environmental genomes, with the exception of two isolates from human clinical cases (genomes HS5256 and HS61650). The environmental samples of this serotype were from the litter and the fly traps collected from the chicken farms. The genomes of *S.* Rissen clustered based on the source of isolates (Fig. 4.5; Appendix 17). The swine fecal genomes were grouped together, while the soil and lagoon genomes even collected from the different farms and time points still clustered together and separated from swine branch.

4.3.3 AMR Correlation based on phenotypic (MIC) and genotypic data (WGS)

Genome sequence data were correlated with the phenotypic AMR profiles to evaluate the ability of WGS to predict phenotypic resistance (Fig. 4.6, Fig. 4.7). The most frequent AMR phenotypes were streptomycin (STR; 57.5%), tetracycline (TET; 51%), and sulfisoxazole (FIS; 46%) (Table S1). Resistance to azithromycin, ciprofloxacin and nalidixic

acid was not detected in this study and, therefore, not included for evaluation. Overall, phenotypic resistance correlated strongly with the presence of corresponding AMR determinants using WGS (Table 4.2). The overall sensitivity of AMR coding genes presence for predicting resistance across all antimicrobials was 87.61%, the specificity was 97.13%, the positive predictive value (PPV) was 88.35%, and the negative predictive value (NPV) was 96.93% as shown in Table 4.2. The genotypic prediction of phenotypic resistance to sulfisoxazole (FIS), tetracyclines (TET), and cephems (ceftriaxone, CRO; cefoxitin, FOX; ceftiofur, XNL) had a sensitivity over 90%, while the other sensitivity values for other antimicrobials was lower than 90%. The genotype prediction of phenotypic resistance to all antimicrobials, other than streptomycin (STR), had specificity greater than 91% (Table 4.2).

4.3.4 Detection of AMR genes, plasmid replicons, and virulence genes using WGS

The WGS data was used to detect the presence and absence of AMR genes, plasmid replicon, and virulence genes in the 200 *Salmonella* genomes (Fig. 4.6, Fig. 4.7). Overall, the most common resistance genes detected were *sul*1 (32.5%), *tet*R (28.5%), and *tet*A (24%) (Table S2). The three most frequent replicons, including ColRNAI, IncFIB, and IncFII were detected in 43%, 16%, and 15.5% of all *Salmonella* sequences, respectively (Table S2). In addition, the 200 *Salmonella* genomes were also screened for virulence genes. One hundred and seventy-five virulence genes were detected in this study using WGS (Table S2). All 200 isolates were positive for thirty-nine virulence genes, including *inv*A, *sip*B, *prg*H, *spa*, *org*A, *iro*N, *sif*A, and *sop*B (Table S2).

4.3.5 Association of AMR genes, plasmid replicons, and virulence genes with different *Salmonella* serotypes using WGS

Serotypes were found to vary with regard to the presence/absence of AMR coding gene, plasmid replicon, and virulence gene using WGS approach based on the odds ratio to evaluate their associations (Table 4.3; Appendix 8). The significant ($P < 0.05$) associations between *S*. Typhimurium and *S*. 4,[5],12:i:- with AMR genes were observed, including *aad*A25, *sul*1, *tet*A, and *tet*G, while the *aad*A1, *aad*A2, *tet*A, and *tet*R genes were found significantly associated with *S*. Derby (Table 4.3). On the other hand, the different AMR genes including *aph*(3")-Ib, *aph*(6)-Id, *str*A, and *str*B were significantly detected in *S*. Schwarzengrund (Table 4.3). Several significant ($P < 0.05$) associations between plasmids and *Salmonella* serotypes were also observed, including IncFIB and IncFII in serotype Typhimurium, 4,[5],12:i:-, and Schwarzengrund, while IncQ2 was significantly found in serotype Derby.

As highlighted previously, several major virulence genes were detected in all *Salmonella* isolates in our study (Table S2). However, *pef*A, *spv*B, and *ssp*H1 were specifically detected in serotype Typhimurium and 4,[5],12:i:- (Table 4.3). Among serotype Typhimurium and 4,[5],12:i:- isolates, the *pef*A, *spv*B, *ssp*H1 together with *rck* virulence genes were significantly associated with the *Salmonella* genomes isolated from human and environmental sources. *S.* Schwarzengrund genomes were significantly associated with the presence of *cdt*B, *iuc*, *iut*A and *spv*B genes, while *gtr*A and *sse* genes were significantly detected in *S*. Derby (Table 4.3).

4.4 Discussion

The objective of this study was to characterize *Salmonella* serotype, AMR determinants and virulence genes using whole genome sequencing. The 200 *Salmonella* genomes were isolated from different sources of origin including human, swine, poultry, and environment, and were analyzed using the core genome SNP-based analysis and the alignment-free analysis method FFP. The phylogenetic trees obtained from parSNP and FFPry showed the clusters based on *Salmonella* serotype (Fig. 4.1). The branch length in FFP is more representative of differences over the whole genome, which may be due to differential plasmid, prophage content, or other accessory genome (27), while SNP utilized the core genomes derived from whole-genome alignment and read mapping for phylogeny construction (26, 27). The major difference of the SNP- and FFP-based analyses was in the order of the serotype clusters within the tree, however, the overall approach selected had relatively little effect on the topology of the phylogenetic trees (Fig. 4.1). A number of studies have reported the use of SNP-based analysis as a potential molecular subtyping tool for outbreak investigation in multiple *Salmonella* serotypes including Dublin (28), Enteritidis (20, 29), Heidelberg (30), Manhattan (19), Montevideo (31, 32), and Typhimurium (33-35). The phylogenetic analysis based on WGS-derived SNPs has been shown to provide greater cluster resolution than the gold standard subtyping method, pulsed-field gel electrophoresis (PFGE), resulting in discrimination of outbreak-related human clinical isolates and food or environmental origins (19-21). In concordance with our study, the SNP trees of individual *Salmonella* serotype including Typhimurium and 4,[5],12:i:- (Fig. 4.2), Schwarzengrund (Fig. 4.4), and Rissen (Fig. 4.5) were mostly clustered based on source of origin. However,

there were some exceptions in each individual tree. As shown in Fig. 4.2, some human Typhimurium genomes (HS71549, HS51537, and HS51628) were found closely related to the environmental, swine, and chicken genomes, respectively. The Schwarzengrund cluster in Fig. 4.4 showed that the genomes from chicken feces clustered with the genomes obtained from environmental isolates which were derived from the same farm. These findings can point towards the potential transmission of *Salmonella* between the chicken and the environment. In contrast, there was no source-dependent clustering in *S.* Derby (Fig. 4.3), with genomes from human, swine and environment mixed. This serotype has a highly homogeneous genetic composition which can be carried by different hosts (36). However, a recent study in China reported that the clustered regularly interspaced short palindromic repeats (CRISPRs) could be a useful subtyping tool for *S.* Derby in molecular epidemiological investigations (37). Though the SNP typing is the reliable tool for genomic and epidemiologic studies, it is not without limitations. SNP-based analysis requires alignment of whole genome sequences and only utilizes the core genome, which may be less sensitive as a result. In addition, this method is still limited to the intragenus analysis of closely related species and strains (26, 38).

The FFP phylogenetic clustering is an effective tool that relies on an alignment-free approach for genomic evolution study. The advantages of FFP-based analysis are that it is independent of a reference genome, and has lower hardware requirements. Additionally, FFP analysis can be performed with whole genome shotgun samples as it is not affected by contig orientation, and contig order (27). FFP-based analyses has been reported in a number of bacterial genomic studies, including *Helicobacter pylori* (27), *Bacillus spp.* (39), *Escherichia*

coli (40, 41), and *Shigella* (41). These studies have revealed that the FFP method can contribute to the phylogenetic clusters based on geographic relation and outbreak detection. Our study is the first to utilize the alignment-free FFP analysis in *Salmonella* and compare it to the core genome SNP-based analysis. We found that the phylogenetic clusters from these methods were similar in term of serotype characterization, but the branching varied due to differences in analysis approaches (Fig. 4.1). While SNP- and MLST-based methods are likely to continue to be the default choice for subtyping and comparative genomics in *Salmonella,* the FFP method can serve as a useful alternative method requiring relatively low-powered computing resources (27).

In this study, we have shown that WGS is an excellent tool for accurately predicting antimicrobial resistant phenotype in human, animal, and environment associated multiple *Salmonella* serotypes, as WGS predictions and phenotypic resistance matched well with high sensitivity and specificity in our study. Overall, the resulting resistance genotypes correlated with 87.61% sensitivity and 97.13% specificity to the resistance phenotype (Table 4.2). Among the discordant results in our study, the lowest specificity of WGS-based AMR prediction was observed for streptomycin which accounted for the presence of streptomycin-resistance genes but lacked phenotypic resistance. This finding was in concordance with the previous studies in *Salmonella* (22, 42) and *E. coli* (23, 43). The streptomycin discrepancies have been commonly detected in other studies too because streptomycin is not used to treat enteric infections, and as such, results in the absence of clinical breakpoint for streptomycin susceptibility in *Salmonella* and *E. coli* (22). Although the *str*A/*str*B and *aad*A genes were detected, the *str*A/*str*B genes conferred higher resistance than *aad*A genes (23, 44). Thus, the

presence of *aad*A genes by *in silico* method may not result in streptomycin resistance phenotypically. In addition, the mechanism of streptomycin resistance is frequently due to mutations in the 16S rRNA gene leading to difficulty of phenotypic prediction (43, 45). Our results suggest that the refinement of WGS-based AMR prediction could be beneficial and can definitely enhance the monitoring of AMR strains and determinants detected in humans, foods, animals, and environment.

The *Salmonella* serotypes significantly correlated with the presence/absence of AMR genes, plasmid replicons, and virulence genes. We observed specific AMR genes in each *Salmonella* serotype (Table 4.3; Appendix 8). This result along with the phylogenetic relatedness revealed that the type of serotype in discussion had the greatest impact on AMR characterization. Previous studies reported that the human *Salmonella* isolates had a more diverse range of AMR genes, as well as plasmids comparing to the isolates from other sources (18, 42). Additionally, the presence of AMR genes has been shown to be primarily associated with a particular host and is not frequently transmitted among different species (18, 42). Several studies have supported our findings that animal and environment are the important sources of AMR determinants and the transmission could occur by the mean of horizontal gene transfer (11, 46, 47). Multiple plasmid replicons were detected in this study using WGS method (Table S2). Plasmids were observed specific to *Salmonella* serotype that was very similar to the AMR genes (Table 4.3). This is in accordance to our previous study that the plasmid profiles were characterized based on *Salmonella* serotype and incompatibility (Inc) groups (11). The IncF (both FI and FII) family found across the

different *Salmonella* serotypes in our study is a well-adapted and commonly distributed plasmid among members of the *Enterobacterceae* family (48, 49).

Multiple virulence genes were identified among the various *Salmonella* serotypes across different sources by WGS (Table S2). These genes have been described to be involved in several processes important for *Salmonella* transmission and infection, including adhesion, type III secretion system (T3SS), host recognition/invasion, filamentous formation, magnesium uptake, iron acquisition, and regulation of stress factors. Our data showed that *Salmonella* isolates recovered from animal or/and environmental sources contained the same virulence genes as carried by human clinical isolates. Along with the phylogenetic analysis, these findings support our view that the high frequency of virulence genes detected in food animal and environment may be transmitted and cause infections in humans (50-52). One of the typhoid-associated virulence factors, the cytolethal distending toxin *cdt*B, was detected in all isolates of sereotype Schwarzengrund, Johannesburg, and Muenster (Table S2), which was similar to a previous study that detected this gene in *S.* Schwarzengrund (53). The *cdt*B is known to produce the typhoid toxins of *S.* Typhi and is not reported from a wider distribution among non-typhoidal *Salmonella* serovars (NTS) (50, 53). However, there were a few reports of the prevalence of this virulence gene in several NTS, including Javiana (50), Montevideo, Schwarzengrund, and Bredeney (53). This data suggested that the *cdt*B toxin may contribute to the pathogenicity in human and animal.

In conclusion, WGS is a helpful tool to assess the phylogenetic relations among multiple serotypes, AMR and virulence gene evaluation and assist in the molecular epidemiological studies of foodborne pathogens. The SNP-based and FFP-based analysis

provided the higher resolution *Salmonella* phylogenetic trees that could differentiate the isolates recovered from human, animal, and environment when compared to PFGE. In addition, WGS is a useful tool for AMR prediction, plasmid replicon, and virulence gene detections. Our study revealed the close relationship of *Salmonella* isolates associated with different hosts among multiple serotypes and the prevalence of AMR genes, plasmid replicons and virulence genes that were identical in different species and could potentially highlight exchange of serotypes across different hosts.

4.5 Materials and methods

4.5.1 *Salmonella* Isolates Selection

The 200 *Salmonella* isolates representing multiple serotypes collected from multiple sources, including human, swine, poultry, and environment, were selected for WGS in our study (Table 4.1). The serotypes were selected across multiple time representing between years 2009-2016. The human *Salmonella* isolates were from stool samples of patients with clinical cases received from the North Carolina State Public Health Laboratory (*n*=44). Swine isolates (*n*=32) originated from fecal, lymph nodes, and carcass swab samples from commercial swine farms in North Carolina, while poultry isolates (*n*=22) were from chicken fecal samples collected from sustainable farms in North Carolina and Tennessee. Environmental isolates (*n*=102) were collected from commercial swine farms and sustainable farms in North Carolina and Tennessee. The list of isolates and details were tabulated in Table S1. All samples were stored in Brucella broth at -80˚C until further characterization.

4.5.2 Phenotypic Antimicrobial Resistance Testing

The antimicrobial susceptibility and the minimum inhibitory concentration (MIC) profile of each *Salmonella* isolate was determined by the broth microdilution method using the gram-negative Sensititre™ (CMV3AGNF) plate (Trek Diagnostic Systems, OH) in accordance with the guidelines and interpretations published by the Clinical and Laboratory Standards Institute (CLSI) and National Antimicrobial Resistance Monitoring System (NARMS). The panel of 15 antimicrobials tested include amoxicillin/clavulanic acid (AMC, suppliers abbreviation AUG; 1/0.5-32/16 μg/ml), ampicillin (AMP; 1–32 μg/ml), azithromycin (AZI; 0.12-16 μg/ml), cefoxitin (FOX; 0.5–32 μg/ml), ceftiofur (XNL; 0.12–8 μg/ml), ceftriaxone (CRO, suppliers abbreviation AXO; 0.25–64 μg/ml), chloramphenicol (CHL; 2–32 μg/ml), ciprofloxacin (CIP; 0.015–4 μg/ml), gentamicin (GEN; 0.25–16 μg/ml), kanamycin (KAN; 8–64 μg/ml), nalidixic acid (NAL; 0.5–32 μg/ml), streptomycin (STR; 2–64 μg/ml), sulfisoxazole (FIS; 16–256 μg/ml), trimetroprim/sulfamethoxazole (SXT; 0.12/2.38-4/76 μg/ml), and tetracycline (TET; 4–32 μg/ml). Internal quality control was performed by the inclusion of *E. coli* ATCC25922. The *Salmonella* isolates with MIC in the intermediate level were categorized into susceptible to avoid overestimation of resistance.

4.5.3 Genome sequence assembly

Genomes were assembled using SPAdes 3.10.1 (54), with contigs < 200 bp and coverage <10-fold excluded from downstream analyses. The assemblies were checked for quality parameters (genome size, largest contig, N50 and L50 values) using QUAST v. 4.5 (25).

4.5.4 *Salmonella* Serotyping and *Salmonella In Silico* Typing Resource (SISTR)

The animal and environmental *Salmonella* isolates were initially sent to the National Veterinary Services Laboratories (NVSL) at Ames, Iowa for serotyping using the Kauffman-White scheme, while the human serotype identification was conducted at the North Carolina State Public Health Laboratory. All 200 *Salmonella* genomes were analyzed using the *Salmonella In Silico* Typing Resource (SISTR) software (https://lfz.corefacility.ca/sistr-app/) for serotype prediction. The SISTR module utilizes O (somatic) antigen, H (flagellar: H1 and H2) antigen, and/or serogroup-specific probes particularly designed for *Salmonella* Genoserotyping Array (SGSA) (24). The results from SISTR interpretation were compared to the traditional Kauffman-White serotyping and categorized into fully matched, ambiguous (multiple serotypes indicated), or of partial prediction (information was missing in overall prediction, but the individual parts provided were correct), incongruent with the reported phenotypic serotype due to the carriage of antigenic determinants (either H1, H2 or O antigen genes) that were not expressed phenotypically, and overall incorrect. The serotype prediction was confirmed by phylogenetic analysis using core genome parSNPs and FFP analysis as described below.

4.5.5 Alignment-free Feature Frequency Profiling and core genome SNPs analysis

The 200 *Salmonella* genomes were identified for core genome SNPs and were clustered using parSNP program from the Harvest suite (26). FFP was performed with the FFP version 3.19 suite of programs (http://sourceforge.net/projects/ffp-phylogeny/) (55, 56), utilizing the FFPry and generated phylogenetic tree (27). Treegraph v2 (57) and Figtree

(http://tree.bio.ed.ac.uk/software/figtree/) were used to annotate and visualize the phylogenetic trees.

4.5.6 Determination of *Salmonella* virulence, plasmid replicons, and antimicrobial susceptibility determinants

The genotyping by *in silico* method for 200 *Salmonella* sequences were done by assembling and annotating via Prokka v1.12 (58). The contigs submitted to PlasmidFinder (59), and ResFinder (60) modules to determine the existing plasmid replicon types, and AMR genes, respectively. Virulence genes were identified with an in-house workflow using SRST2 v0.1.4.5 (61). The Illumina raw reads were mapped against chromosomal and plasmid virulence genes found in the Virulence Factor Database for *Salmonella* (VFDB) which currently contains 2,017 genes database associated with virulence in *Salmonella* [http://www.mgc.ac.cn/VFs/status.htm] (62). Finally, the presence/absence of AMR determinants, plasmid replicons, and virulence genes were calculated for association with *Salmonella* serotype using odds rations along with Chi-square test or Fisher's exact test with the *P*-value level < 0.05 of significance.

4.5.7 Correlation of susceptibility phenotypes and genotypes

All phenotypic characters were generated from the 200 *Salmonella* isolates by broth microdilution (Sensititre™) antimicrobial susceptibility testing previously described. Each interpretation of resistant or susceptible to a given antimicrobial drug were compared to the presence or absence of a known corresponding resistance genes and/or specific structural gene mutations detected by the WGS. The isolates interpreted as intermediate level were considered as susceptible in this analysis. The percentage of correlation between resistance

phenotypes and genotypes were calculated. The phenotypic results were counted as the reference outcome, sensitivity was calculated by dividing the number of isolates that were genotypically resistant by the total number of isolates exhibiting clinical resistance phenotypes. Specificity was also calculated by dividing the number of isolates that were genotypically susceptible by the total number of isolates with susceptible phenotypes. The percentages of positive predictive values (PPV) and negative predictive values (NPV) were calculated as well.

4.5.8 Accession number(s)

Paired-end reads for the 200 *Salmonella* isolates in this study have been deposited in the National Center for Biotechnology Information's (NCBI) under the Bioproject accession number PRJNA293224. Individual Sequence Read Archive (SRA) accession numbers have been tabulated in the separated sheet.

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Table 4.1 Number of *Salmonella* isolates (*n*=200) from human, animal, and environment by serotype sequenced for comparison

Salmonella serotype (*n*)	Source of isolate				
	Human (*n*=44)	Swine (*n*=32)	Poultry (*n*=22)	Env. (*n*=102)	Total (*n*=200)
Altona				11	11
Anatum				1	1
Braenderup				1	1
Chester	1				1
Derby	9	7		5	21
Enteritidis	1				1
Heidelburg	1			1	2
Kentucky			4	1	5
Johannesburg		4		5	9
Mbandaka	1	3	3	5	12
Muenchen	9				9
Muenster				16	16
Ouakam		1			1
Rissen		6		8	14
Schwarzengrund	7		7	8	22
Senftenberg		6	3	3	12
Typhimurium	15	5	1	18	39
4,[5],12:i:-			4	4	8
Uganda				4	4
Worthington				11	11

Table 4.2 Comparison between genotypic AMR prediction by WGS and phenotypic expression based on MIC levels of AMR *Salmonella* isolates (*n*=200)

AM[1]	Resistant by phenotype		Susceptible by phenotype		Sensitivity (%)	Specificity (%)	PPV[2] (%)	NPV[3] (%)
	WGS: AMR gene +	WGS: AMR gene -	WGS: AMR gene +	WGS: AMR gene -				
AMP	36	7	4	153	83.72	97.45	90	95.63
AMC	8	2	4	186	80	97.89	66.67	98.94
CRO	9	1	3	187	90	98.42	75	99.47
FOX	6	1	3	187	90	98.42	75	99.47
XNL	6	1	3	187	90	98.42	75	99.47
GEN	24	4	5	167	85.71	97.09	82.76	97.66
KAN	37	9	9	145	80.43	94.16	80.43	94.08
STR	98	17	10	75	85.22	88.24	90.74	81.52
FIS	87	5	4	104	94.57	96.29	95.6	95.41
SXT	6	1	1	192	85.71	99.48	85.71	99.48
TET	93	9	8	90	91.18	91.84	94.9	88.24
CHL	7	2	1	190	77.78	99.48	87.5	98.96
Overall					87.61	97.13	88.35	96.93

[1] Antimicrobials: ampicillin (AMP), amoxicillin/clavulanic acid (AMC), ceftriaxone (CRO), cefoxitin (FOX), Ceftiofur (XNL), chloramphenicol (CHL), streptomycin (STR), gentamicin (GEN), Kanamycin (KAN), sulfisoxazole (FIS), trimetroprim/sulfamethoxazole (SXT), and tetracycline (TET)

[2] positive predictive value (PPV)

[3] negative predictive value (NPV)

Table 4.3 AMR determinant, plasmid replicon, and virulence gene detections based on WGS in *Salmonella* serotypes

Characteristic:	***S*. Typhimurium & *S*. 4,[5],12:i:-**	***S*. Derby**	***S*. Schwarzengrund**
AMR gene (OR)*	*aad*A25 (11.05) *sul*1 (2.18) *tet*A (0.23) *tet*G (∞)	*aad*A1 (4.22) *aad*A2 (3.2) *tet*A (4.57) *tet*R (10.44)	*aph*(3")-Ib (48.9) *aph*(6)-Id (25.25) *str*A (48.9) *str*B (40.48)
Plasmid (OR)*	colRNAI (5.07) IncFIB (8.4) IncFII (10.51)	IncQ2 (∞)	IncFIB (7.22) IncFIC (∞) IncFII (20.53)
Virulence gene (OR)*	*pef*A (224) *spv*B (268.24) *ssp*H1 (3.28)	*gtr*A (5.38) *sse*J/K1/L (∞) *sse*K2 (4.5)	*cdt*B (∞) *iuc*A/B/C/D (92.27) *iut*A (92.27) *spv*B (0)

* An odds ratio (OR) of 0 indicates the absence of that gene in a given *Salmonella* serotype, while the OR of infinity (∞) indicates that the mentioned gene was detected only in a specific serotype and none of the other serotypes.

Only the odds ratios with *P*-value < 0.05 are shown.

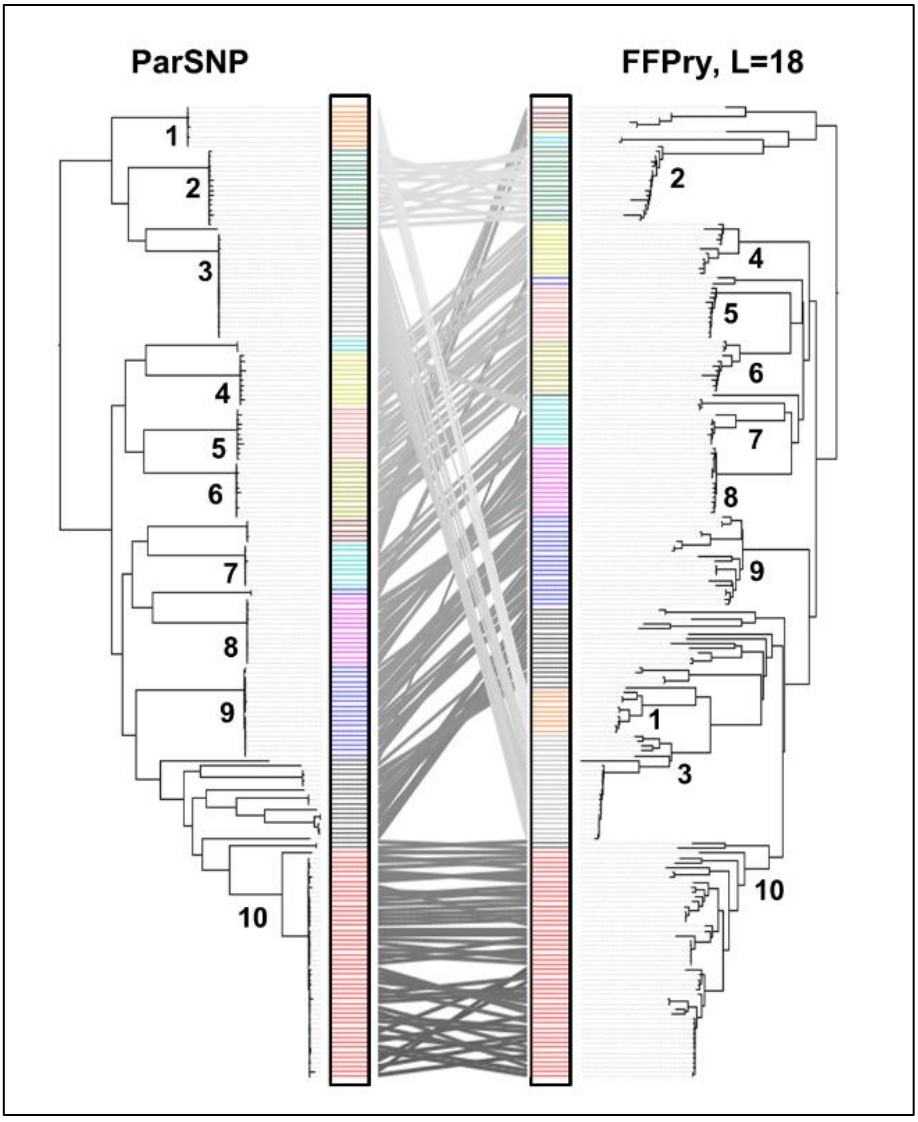


Figure 4.1 Comparison of *Salmonella enterica* phylogenetic trees based on core genome single nucleotide polymorphisms using ParSNP (26) and the alignment-free whole genome comparison with feature frequency profiling of purine-pyrimidine words (FFPry) with a word length (L) of 18 (27), using the 200 *Salmonella* genomes included here,visualised using the serovars (colored bar) and a tanglegram in the middle to indicate position of individual genomes in both phylogenetic trees.

Figure 4.1 Continued

The left panel represents the phylogenetic tree based on core genome SNPs, while the right panel shows the phylogenetic tree obtained FFPry. Note that although the order of serotypes differs, isolates cluster generally according to serotype in both analysis methods, with the overall topology being similar. Major serovars are indicated by numbers: 1. Johannesburg; 2. Muenster; 3. Schwarzengrund; 4. Worthington; 5. Altona; 6. Mbandaka; 7. Senftenberg; 8. Rissen; 9. Derby; 10. Typimurium and 4,[5],12:i:-.

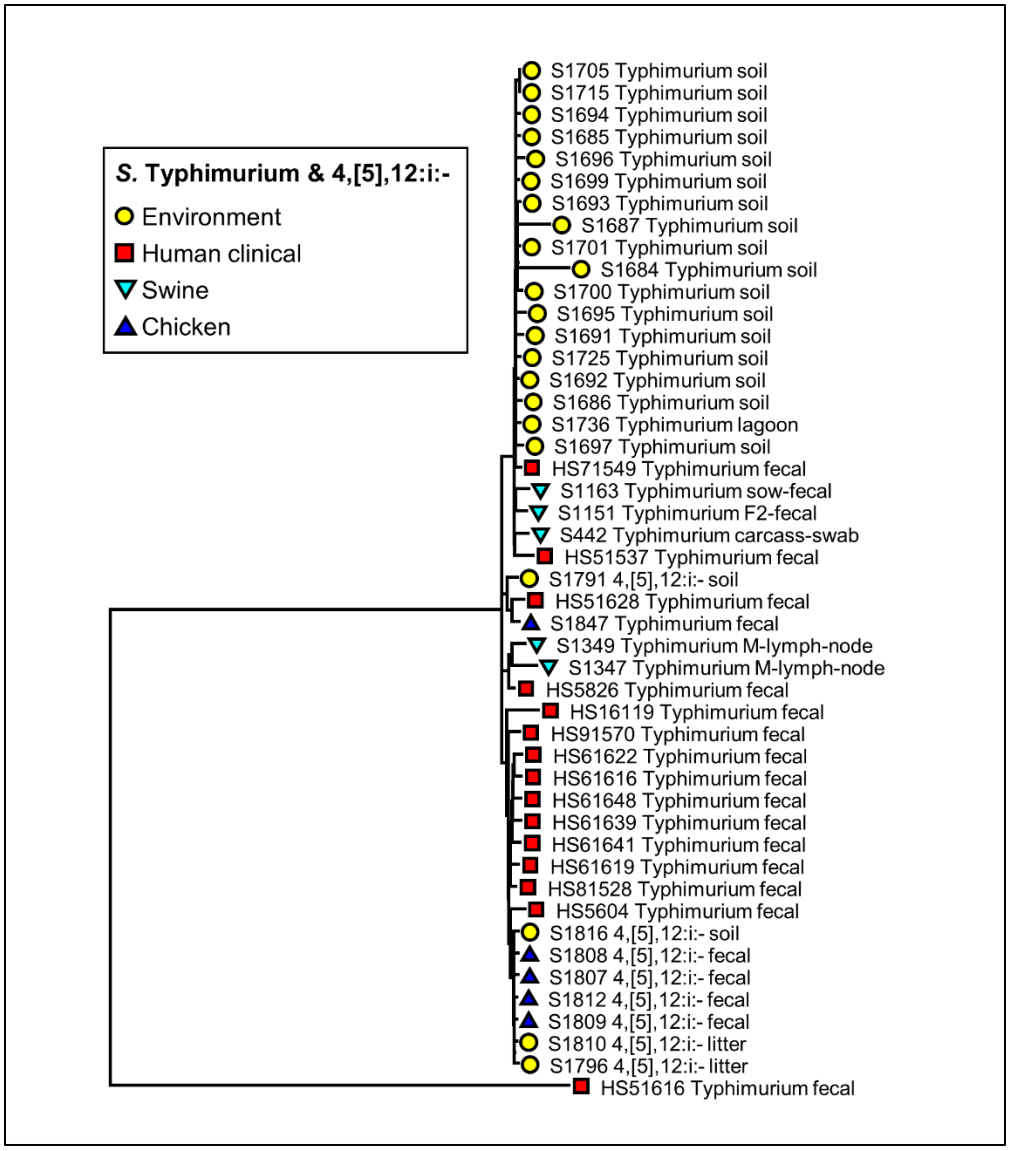


Figure 4.2 Phylogenetic tree of *S.* Typhimurium and *S.* 4,[5],12:i:- isolates (*n*=47) recovered from human, swine, chicken, and environmental sources constructed using parSNP analysis. Colored markers indicate the source of each isolate, with more details added to the name of each isolate.

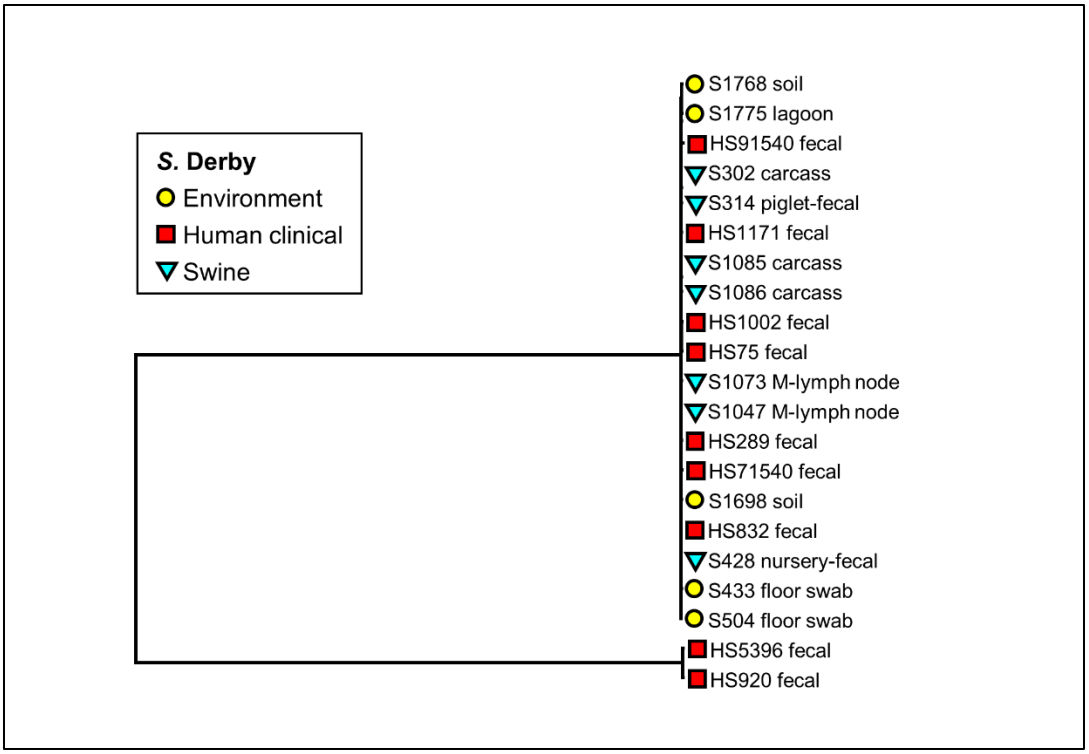


Figure 4.3 Phylogenetic tree of *S.* Derby isolates (*n*=21) recovered from recovered from human, swine, and environmental sources constructed using parSNP analysis. Colored markers indicate the source of each isolate, with more details added to the name of each isolate.

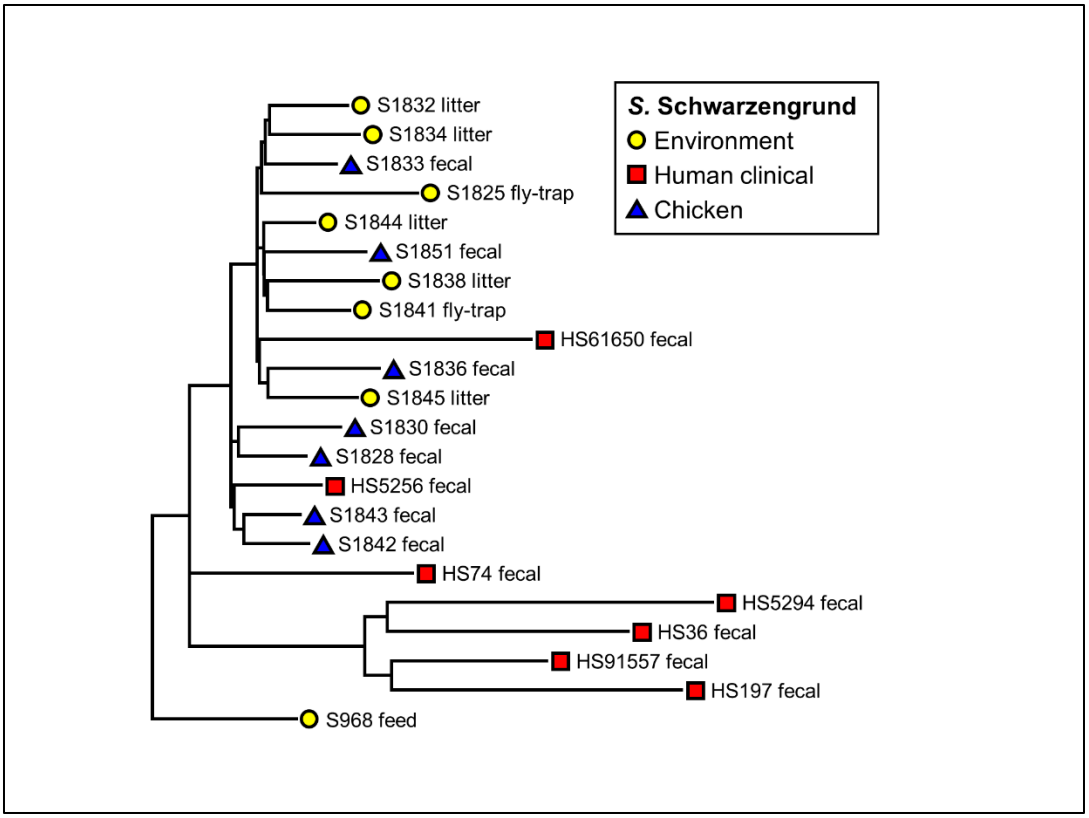


Figure 4.4 Phylogenetic tree of *S.* Schwarzengrund isolates (*n*=22) recovered from recovered from human, chicken, and environmental sources constructed using parSNP analysis. Colored markers indicate the source of each isolate, with more details added to the name of each isolate.

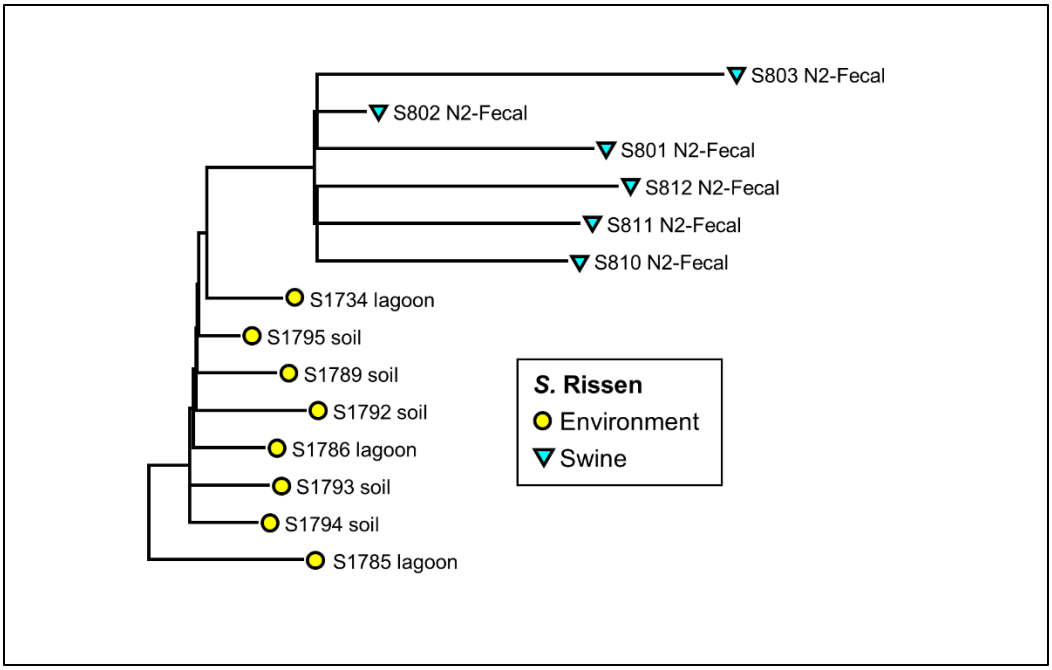


Figure 4.5 Phylogenetic tree of *S.* Rissen isolates (*n*=14) recovered from recovered from swine and environmental sources constructed using parSNP analysis. Colored markers indicate the source of each isolate, with more details added to the name of each isolate.

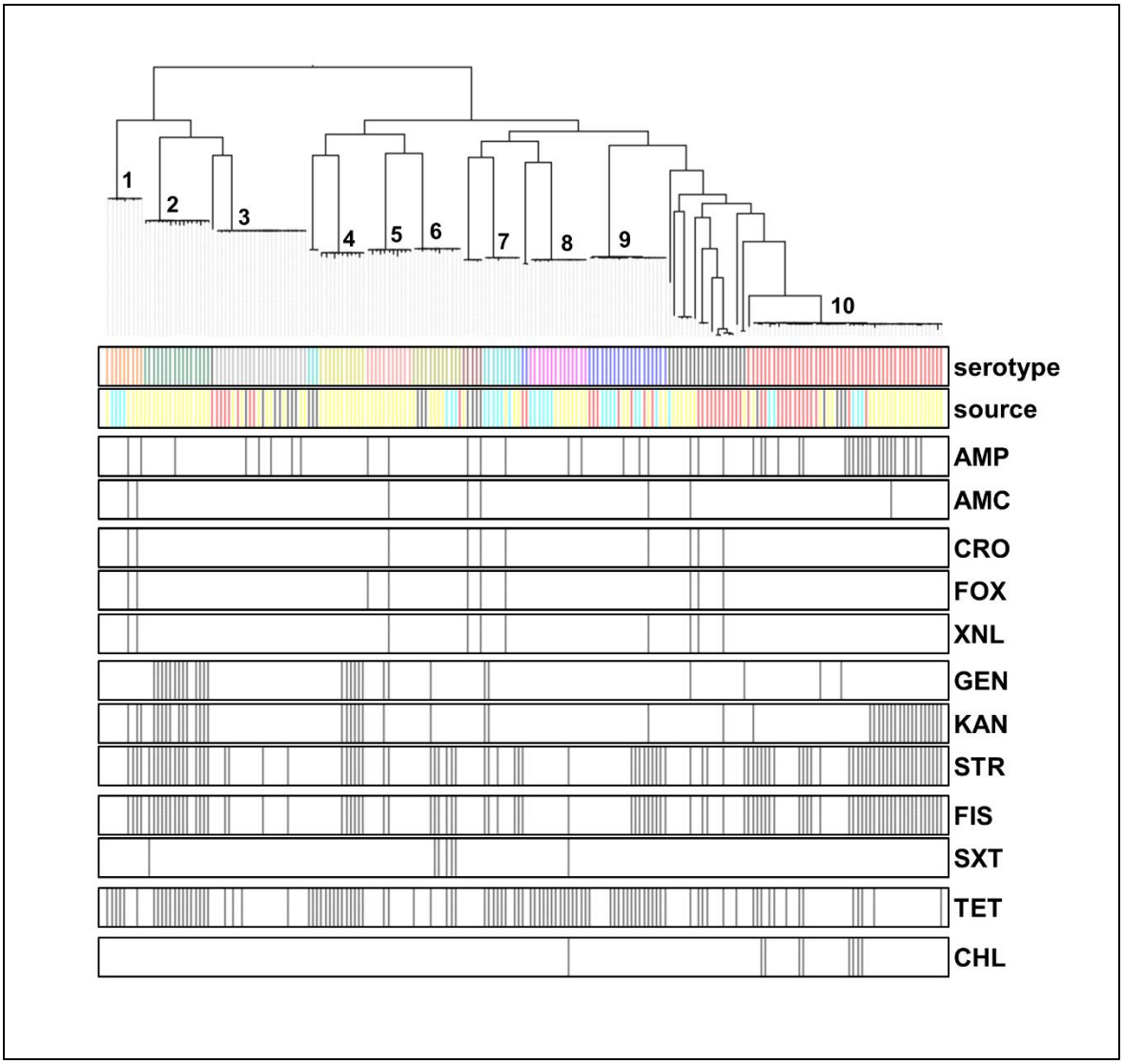


Figure 4.6 Distribution of antimicrobial resistance of the 200 *Salmonella* genomes included in this study. The genomes were clustered based on core genome SNPs using ParSNP (26) and the antimicrobial resistances are shown by black lines in the respective bars below.

Figure 4.6 Continued

Antimicobials used are grouped according to their class and mechanism: penicillins (AMP, ampicillin; AMC, amoxicillin and clavulanate [augmentin, AUG]); cephalosporins (CRO, ceftriaxone (AXO); FOX, cefoxitin; XNL, ceftiofur); aminoglycosides (GEN, gentamicin; KAN, kanamycin; STR, streptomycin); sulfanomides/folate inhibitors (FIS, sulfisoxazole; SXT, trimetroprim and sulfamethoxazole); tetracycline (TET) and chloramphenicol (CHL). For source, these were subdivided into four major classes: environment (yellow), human clinical (red), chicken (dark blue) and swine (light blue). Major serovars are indicated by numbers: 1. Johannesburg; 2. Muenster; 3. Schwarzengrund; 4. Worthington; 5. Altona; 6. Mbandaka; 7. Senftenberg; 8. Rissen; 9. Derby; 10. Typimurium and 4,[5],12:i:-.

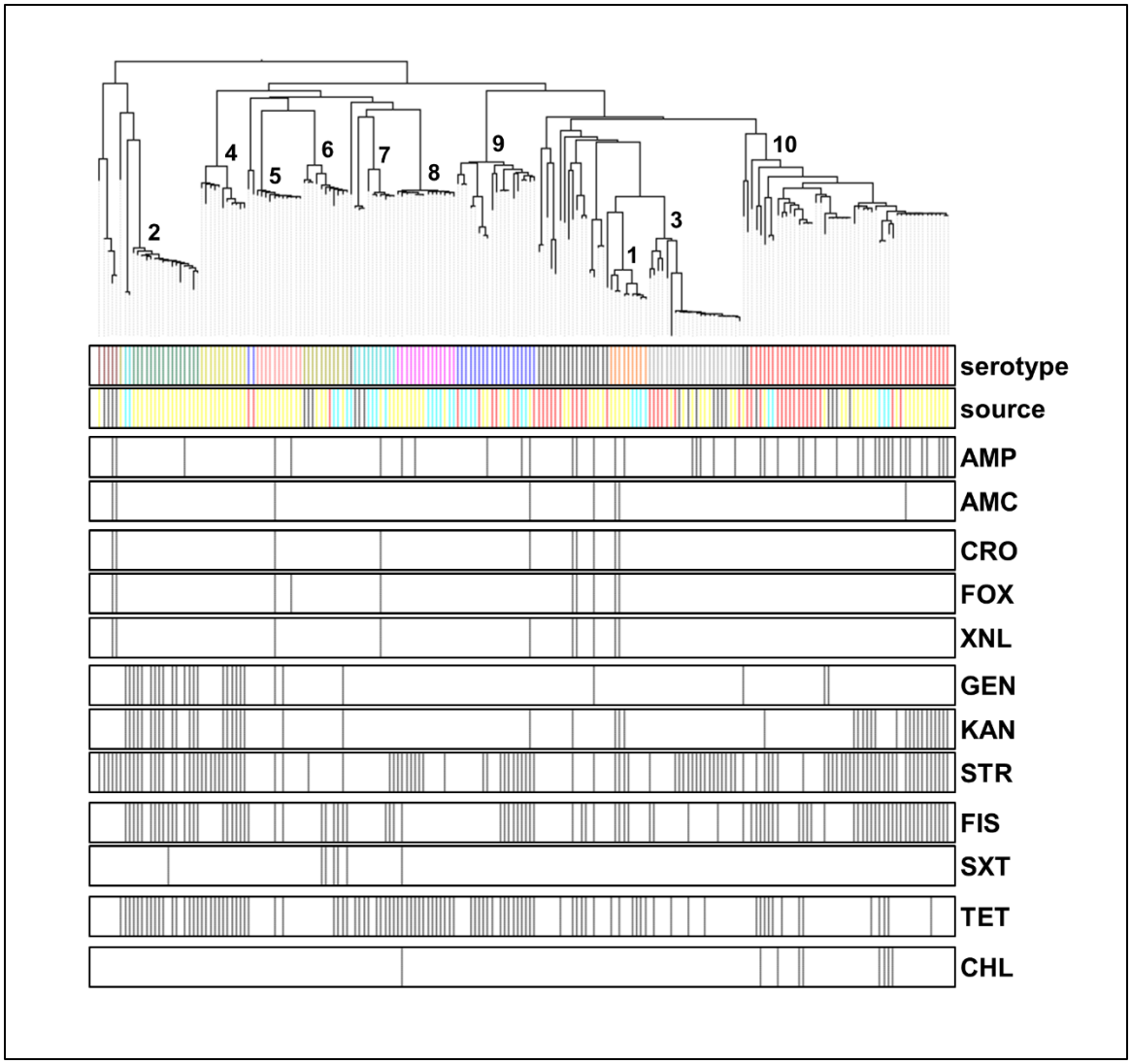


Figure 4.7 Distribution of antimicrobial resistance of the 200 *Salmonella* genomes included in this study. The genomes were clustered based on feature frequency profiling of purine-pyrimidine words (FFPry) with a word length (L) of 18 (27) and the antimicrobial resistances are shown by black lines in the respective bars below (Supplement).

Figure 4.7 Continued

Antimicobials used are grouped according to their class and mechanism: penicillins (AMP, ampicillin; AMC, amoxicillin and clavulanate [augmentin, AUG]); cephalosporins (CRO, ceftriaxone (AXO); FOX, cefoxitin; XNL, ceftiofur); aminoglycosides (GEN, gentamicin; KAN, kanamycin; STR, streptomycin); sulfanomides/folate inhibitors (FIS, sulfisoxazole; SXT, trimetroprim and sulfamethoxazole); tetracycline (TET) and chloramphenicol (CHL). For source, these were subdivided into four major classes: environment (yellow), human clinical (red), chicken (dark blue) and swine (light blue). Major serovars are indicated by numbers: 1. Johannesburg; 2. Muenster; 3. Schwarzengrund; 4. Worthington; 5. Altona; 6. Mbandaka; 7. Senftenberg; 8. Rissen; 9. Derby; 10. Typimurium and 4,[5],12:i:-.

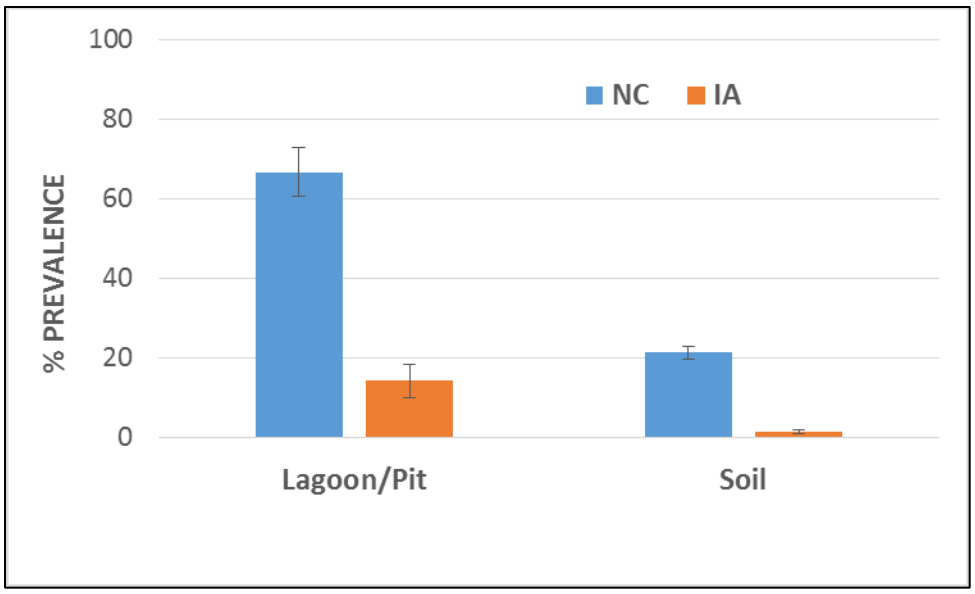
APPENDICES

Appendix 1 *Salmonella* positive isolates from farms in North Carolina and Iowa

Farms*	Frequency (N)	Positive (%)
NCF 1	110	47 (42.73)
NCF 2	110	4 (3.64)
NCF 3	110	59 (53.64)
NCF 4	110	30 (27.27)
NCF 5	110	11 (10.00)
NCF 6	110	17 (15.45)
Total NCF	**660**	**168 (25.45)**
IAF 6	110	21 (19.09)
Total IAF	**770**	**21 (2.73)**
Total all	**1430**	**189 (13.22)**

* NCF: North Carolina Farm, IAF: Iowa Farm

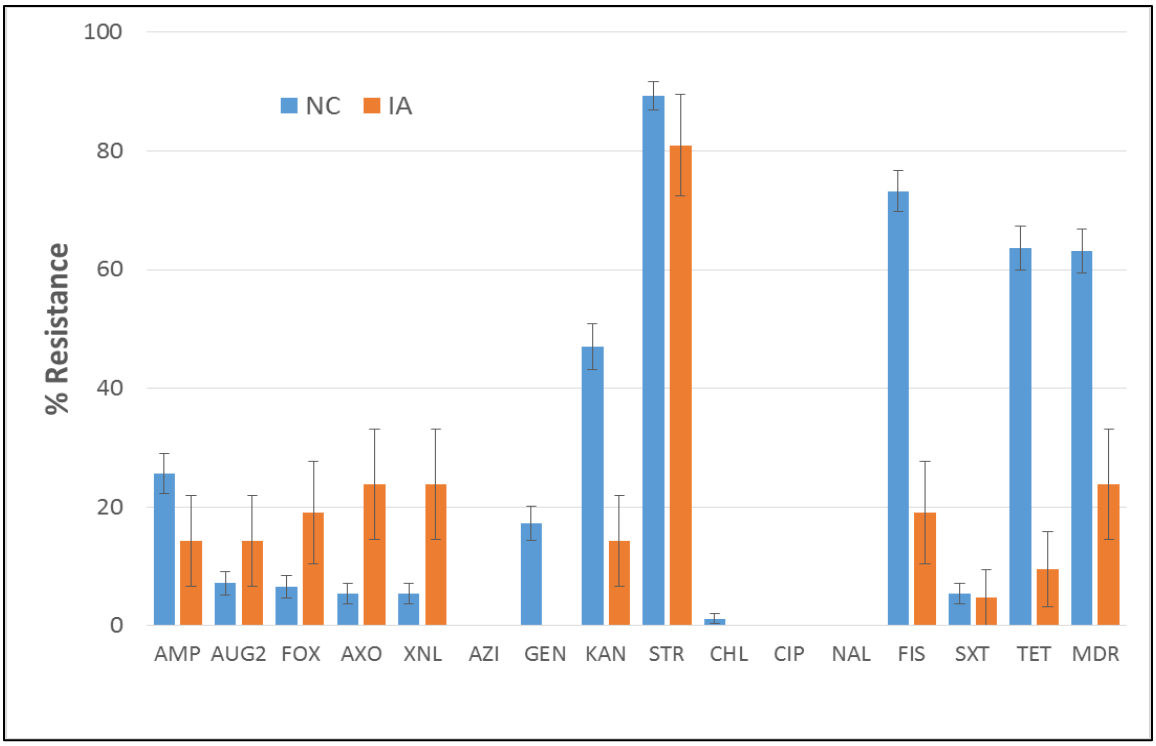
Appendix 2 *Salmonella* prevalence by sample types isolated from North Carolina and Iowa



Appendix 3 The distribution of *Salmonella* serotypes from lagoon/pit and soil samples in North Carolina and Iowa (*n*=189)

***Salmonella* serotype**	**North Carolina** *n* (%)		**Iowa** *n* (%)		**Total** *n* (%)
	Lagoon	**Soil**	**Pit**	**Soil**	
Altona	1 (2.44)	13 (10.24)			14 (7.41)
Anatum			10 (100)	3 (27.27)	13 (6.88)
Derby	1 (2.44)	8 (6.30)			9 (4.76)
Infantis				1 (9.09)	1 (0.53)
Johannesburg	4 (9.76)	4 (3.15)			8 (4.23)
Litchffield				7 (63.64)	7 (3.7)
Mbandaka	1 (2.44)	3 (2.36)			4 (2.12)
Muenster	5 (12.2)	11 (8.66)			16 (8.47)
Ohio	3 (7.32)	4 (3.15)			7 (3.7)
Ouakam		1 (0.79)			1 (0.53)
Rissen	10 (24.39)	7 (5.51)			17 (8.99)
Rough_O:z10:e,n,z15	1 (2.44)				1 (0.53)
Senftenberg	9 (21.95)	19 (14.96)			28 (14.81)
Typhimurium var5-	3 (7.32)	39 (30.71)			42 (22.22)
Uganda	2 (4.88)	2 (1.57)			4 (2.12)
Worthington	1 (2.44)	14 (11.02)			15 (7.94)
4,12:i:-		1 (0.79)			1 (0.53)
6,7:-:e,n,z15		1 (0.79)			1 (0.53)

Appendix 4 The percentage of antimicrobials resistance of *Salmonella* isolated from North Carolina and Iowa farms



ampicillin (AMP), amoxicillin/clavulanic acid (AUG2), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and multidrug resistance (MDR)

Appendix 5 Antimicrobial resistance of *Salmonella* isolates from North Carolina and Iowa with statistical results using Chi-squared/Fisher's exact tests

Antimicrobials[a]		#isolates	% resistance	χ^2statistic (or Fisher's exact[b] when n<5)	*P*-value*
AMP	NC	168	25.6	0.7551	0.3849
	IA	21	14.29		
AUG	NC	168	7.14	F	0.2231
	IA	21	14.29		
FOX	NC	168	6.55	F	0.06825
	IA	21	19.05		
AXO	NC	168	5.36	F	0.0108*
	IA	21	23.81		
XNL	NC	168	5.36	F	0.0108*
	IA	21	23.81		
AZI	NC	168	0	N/A	N/A
	IA	21	0		
GEN	NC	168	17.26	F	0.04843*
	IA	21	0		
KAN	NC	168	47.02	6.8668	0.008781*
	IA	21	14.29		
STR	NC	168	89.29	F	0.2771
	IA	21	80.95		
CHL	NC	168	1.19	F	1
	IA	21	0		
CIP	NC	168	0	N/A	N/A
	IA	21	0		
NAL	NC	168	0	N/A	N/A
	IA	21	0		
FIS	NC	168	73.21	83.4189	<0.0001*
	IA	21	19.05		
SXT	NC	168	5.36	F	1
	IA	21	4.76		
TET	NC	168	63.69	20.2716	<0.0001*
	IA	21	9.52		
MDR	NC	168	63.1	10.3206	0.001316*
	IA	21	23.81		

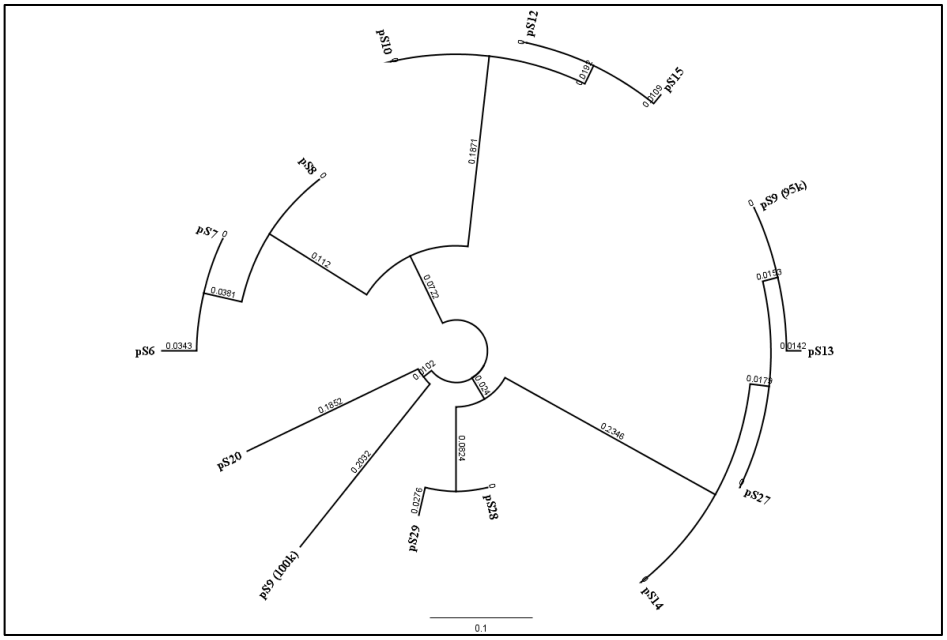
Appendix 5 Continued

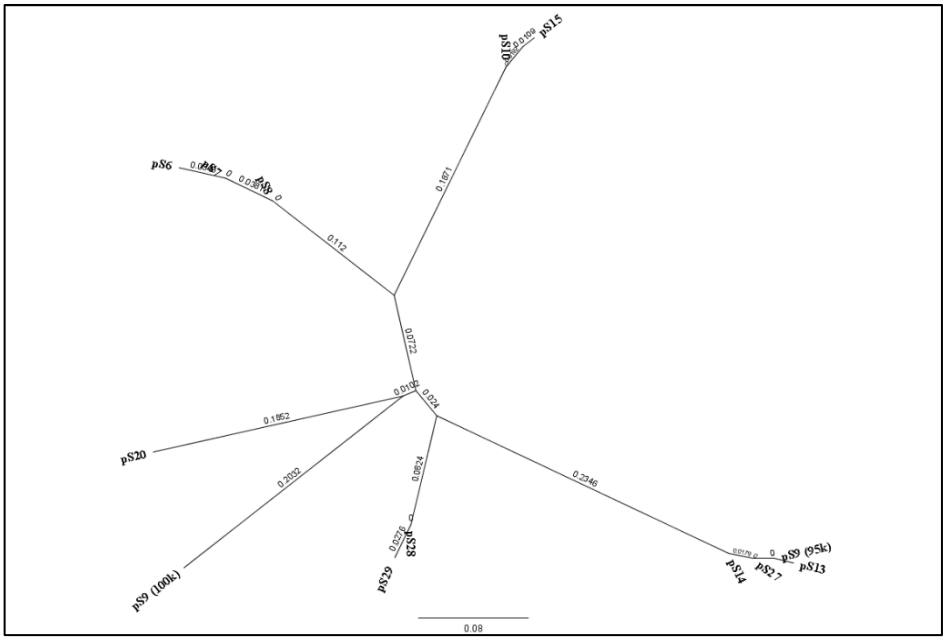
*Chi-square *P*-value lower than 0.05 is considered as significance.

[a] ampicillin (AMP), amoxicillin/clavulanic acid (AUG2), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), kanamycin (KAN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), multidrug resistance (MDR)

[b] F: Fisher's exact test if expected counts < 5.

Appendix 6-7 Phylogenetic diversity for sequences of 14 plasmids acquired from environmental *Salmonella* isolates.





Appendix 6-7 Continued

The evolutionary distances between plasmids were computed using a neighbor-joining algorithm. The distance was obtained from pairwise alignments with 70% similarity and no outgroup. The plasmid label names relate to data in Table 3.1. Phylogenetic analyses were conducted in Geneious R10.

Appendix 8 Association of AMR genes and virulence genes based on WGS with human, animal, and environmental sources of specific *Salmonella* serotypes

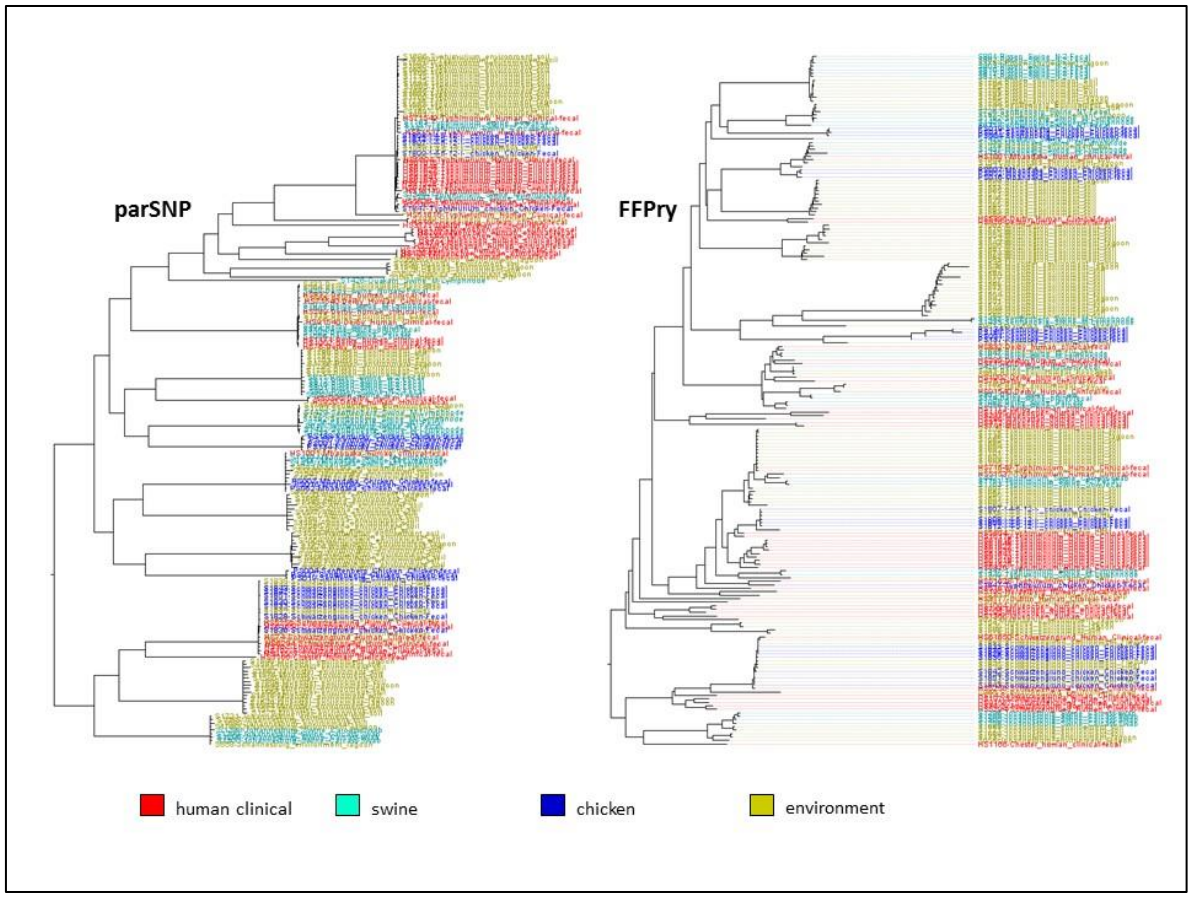
Characteristic	Human (OR)*	Animal (OR)*	Environment (OR)*
S. Typhimurium & *S.* 4,[5],12:i:-			
AMR gene:			
• *aad*A1	0.31	**0.06**	0.15
• *aad*A2	**0.12**	1.16	**3.63**
• *str*A/B	0	9	9
• *sul*1	**0.04**	0.21	**46.45**
• *tet*A	0.69	**15.43**	0
• *tet*G	0.69	**15.43**	0
Virulence gene:			
• *pef*A	**0.12**	1.78	**4.33**
• *rck*	**0.12**	1.78	**4.33**
• *spv*B	**0.08**	1.42	**8.06**
• *ssp*H1	**Infinity**	0	**0**
S. Derby			
AMR gene:			
• *aad*A1	0.5	1	0.5
• *aad*A2	0.5	1	0.5
• *sul*1	0.5	1	2.5
• *tet*A	0.5	1	2.5
Virulence gene:			
• *gtr*A	3.5	0.3	0.9
• *iro*N	4	0.68	0.35
S. Schwarzengrund			
AMR gene:			
• *aph*(3")-Ib	0.1	**Infinity**	0.52
• *aph*(6)-Id	**0.03**	Infinity	3.89
• *str*A	0.1	Infinity	0.52
• *str*B	**0.03**	Infinity	3.89
• *tet*A	2	0	0
Virulence gene:			
• *iuc*A/B/C/D	0.14	Infinity	3.89
• *sop*A	0	Infinity	Infinity

Appendix 8 Continued

*An odds ratio (OR) of 0 indicates the absence of that gene in a given *Salmonella* serotype or no detection of that gene was found in the other serotypes in the study. The OR of infinity (∞) indicates that the mentioned gene was detected only in a given serotype and none of the other serotypes or the mentioned gene was found in all isolates in a given serotype.

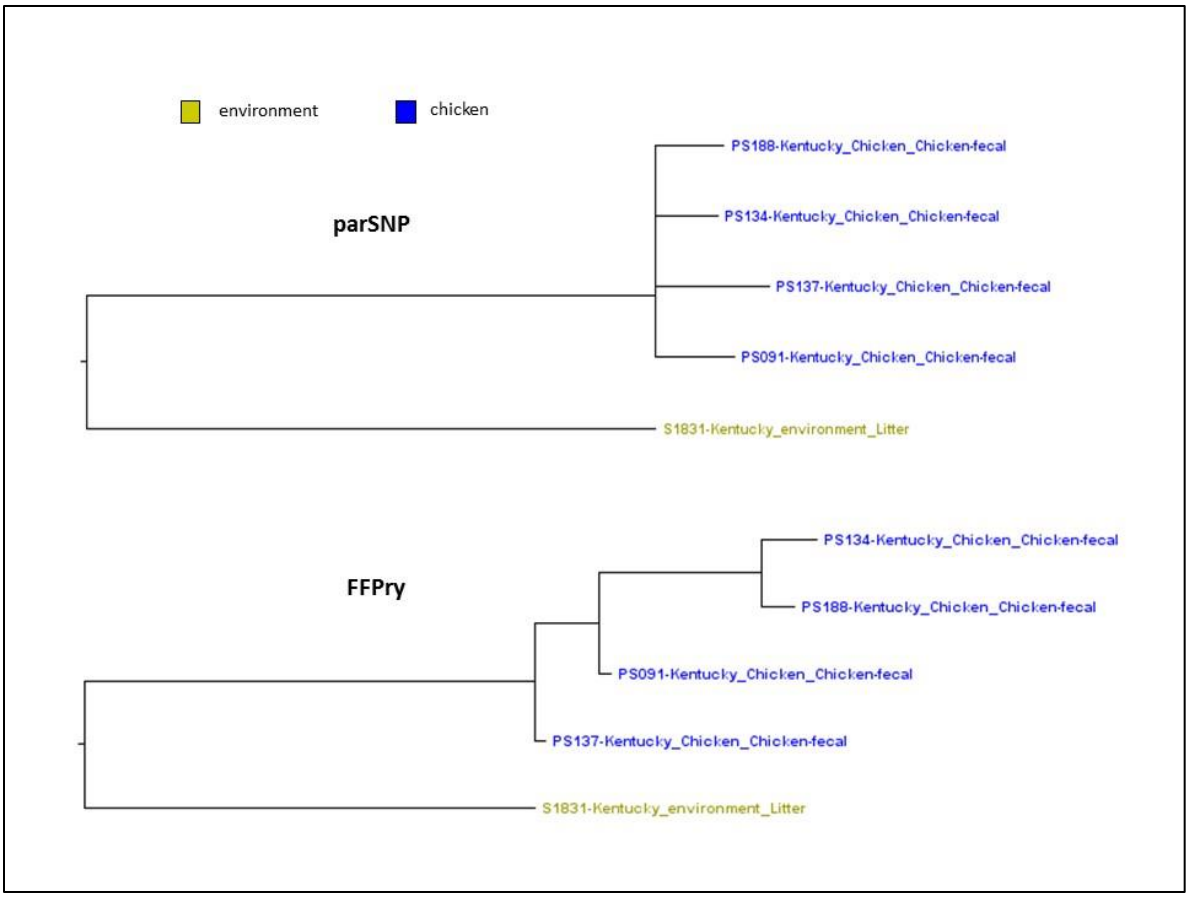
Values in boldface were significant ($P<0.05$).

Appendix 9 Comparison of phylogenetic trees obtained by SNP and FFP with the 200 *Salmonella* genome sequences analysis by the sources of isolate.



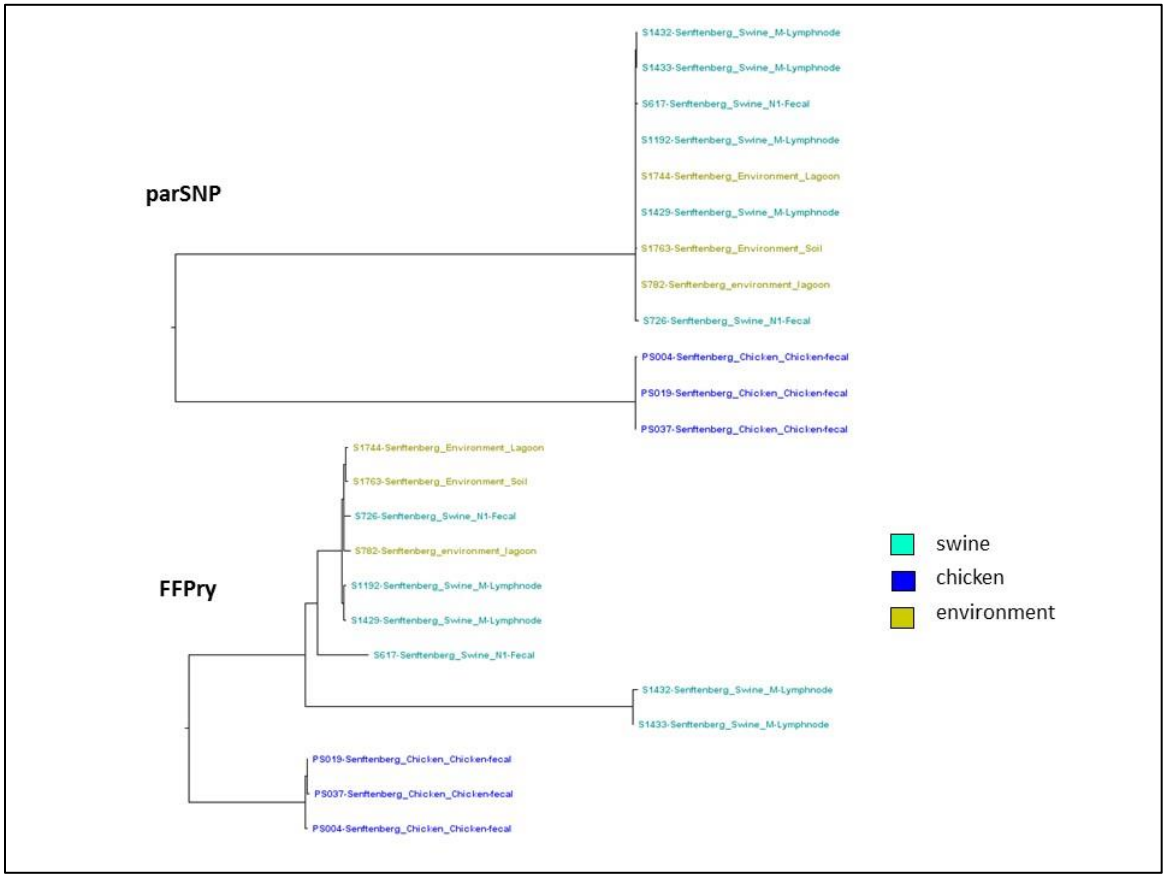
The left panel represents the phylogenetic tree based on SNPs obtained using parSNP; the right panel shows the phylogenetic tree obtained from alignment-free feature frequency profiling of purine/pyrimidine residues (FFPry). Colors indicate each *Salmonella* serotype cluster.

Appendix 10 Phylogenetic tree of *S.* Kentucky isolates (*n*=5) recovered from poultry, and environmental sources constructed using parSNP and FFpry analysis.



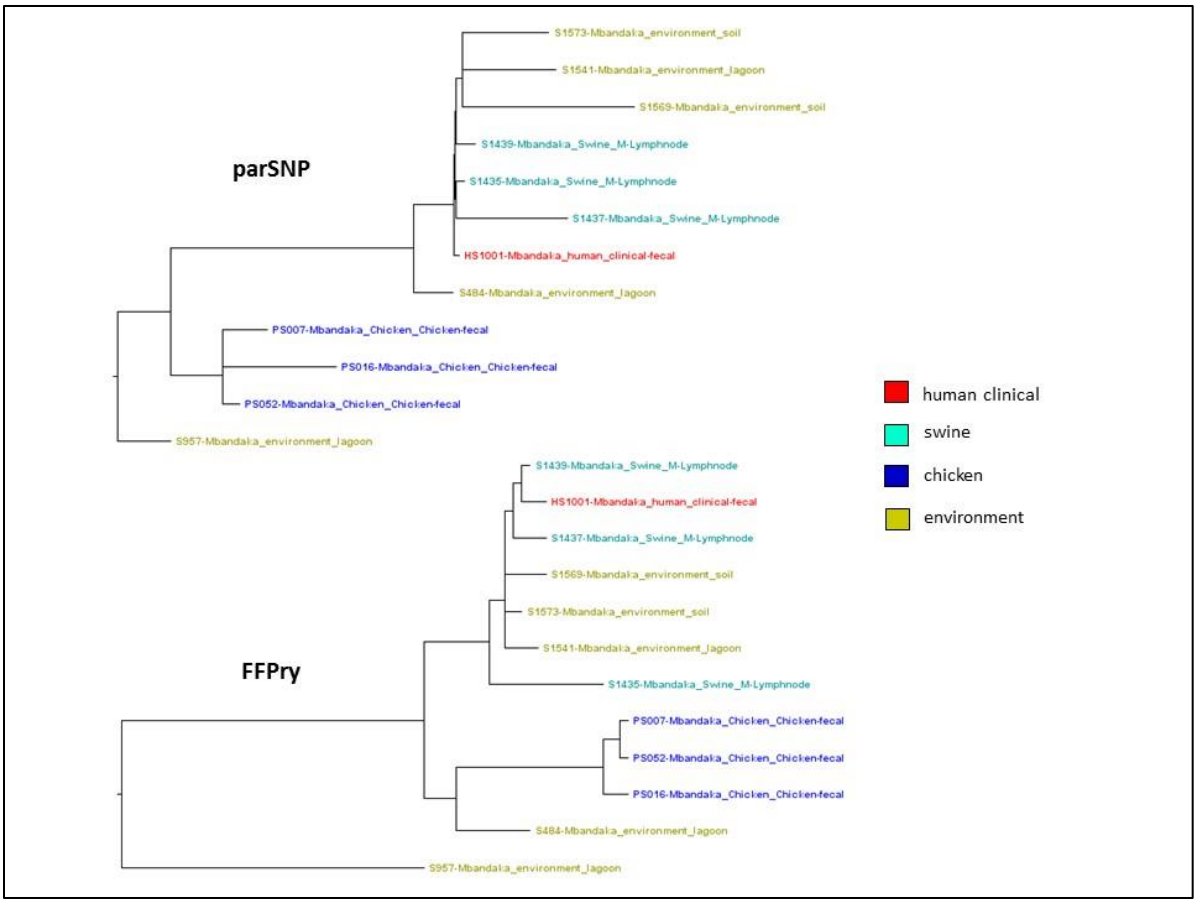
Colors indicate each source of isolate origin.

Appendix 11 Phylogenetic tree of *S.* Senftnberg isolates (*n*=12) recovered from swine, poultry, and environmental sources constructed using parSNP and FFPry analysis.



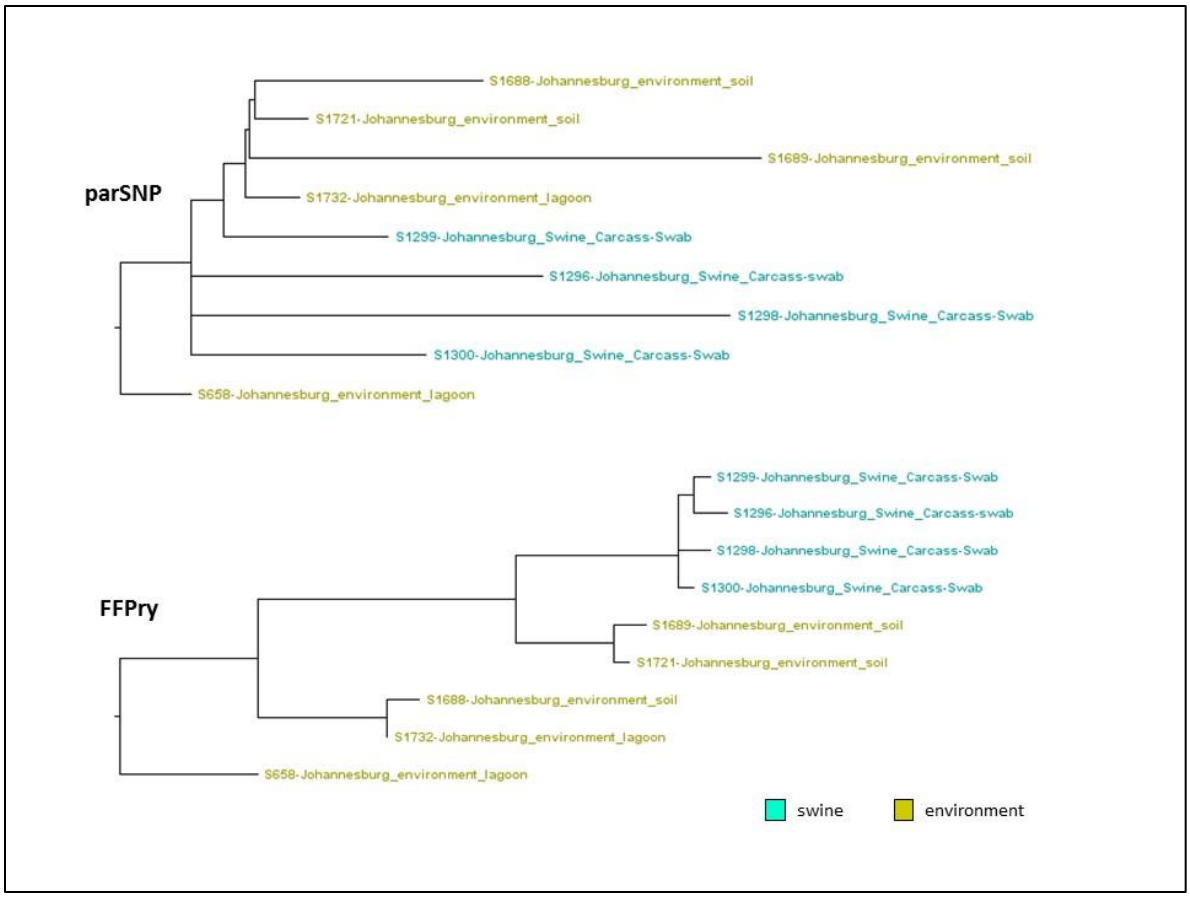
Colors indicate each source of isolate origin.

Appendix 12 Phylogenetic tree of *S.* Mbandaka isolates (*n*=12) recovered from human, swine, poultry, and environmental sources constructed using parSNP and FFPry analysis.



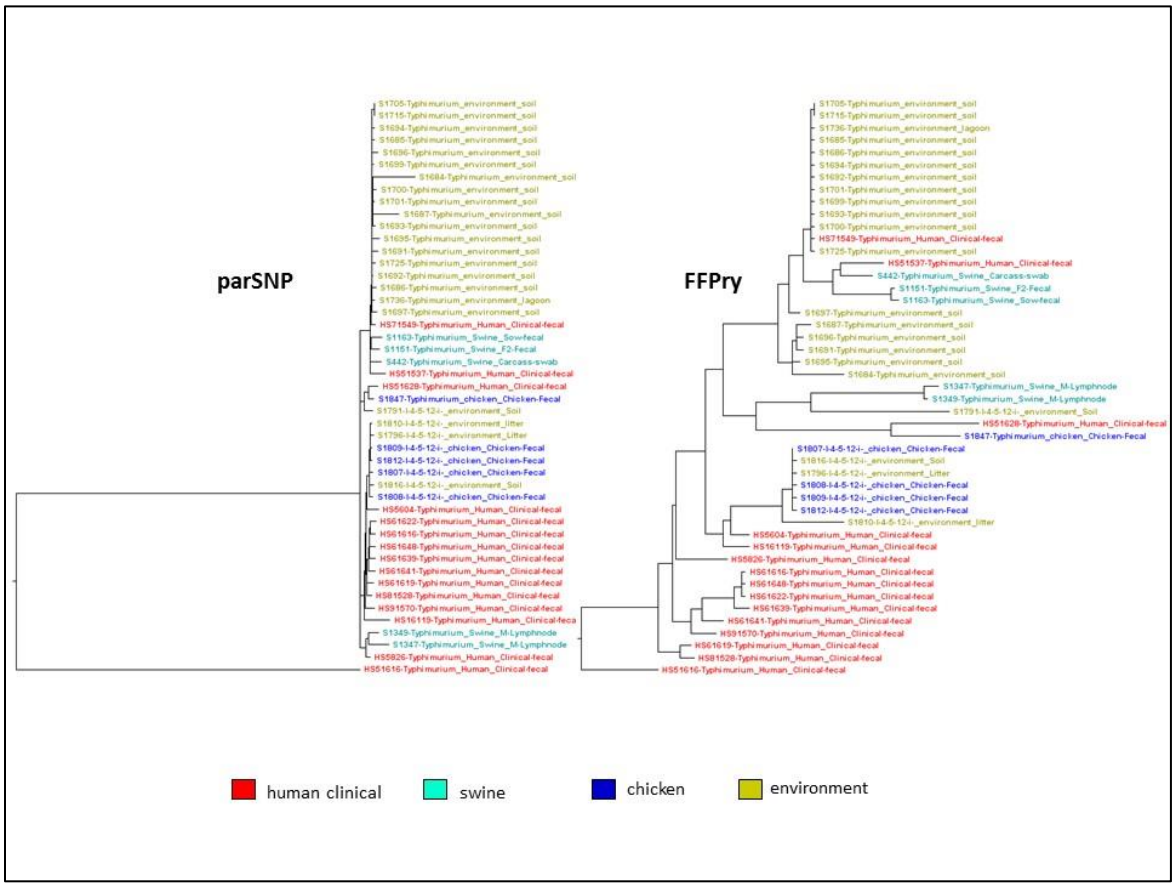
Colors indicate each source of isolate origin.

Appendix 13 Phylogenetic tree of *S.* Johannesburg isolates (*n*=9) recovered from swine, and environmental sources constructed using parSNP and FFPry analysis.



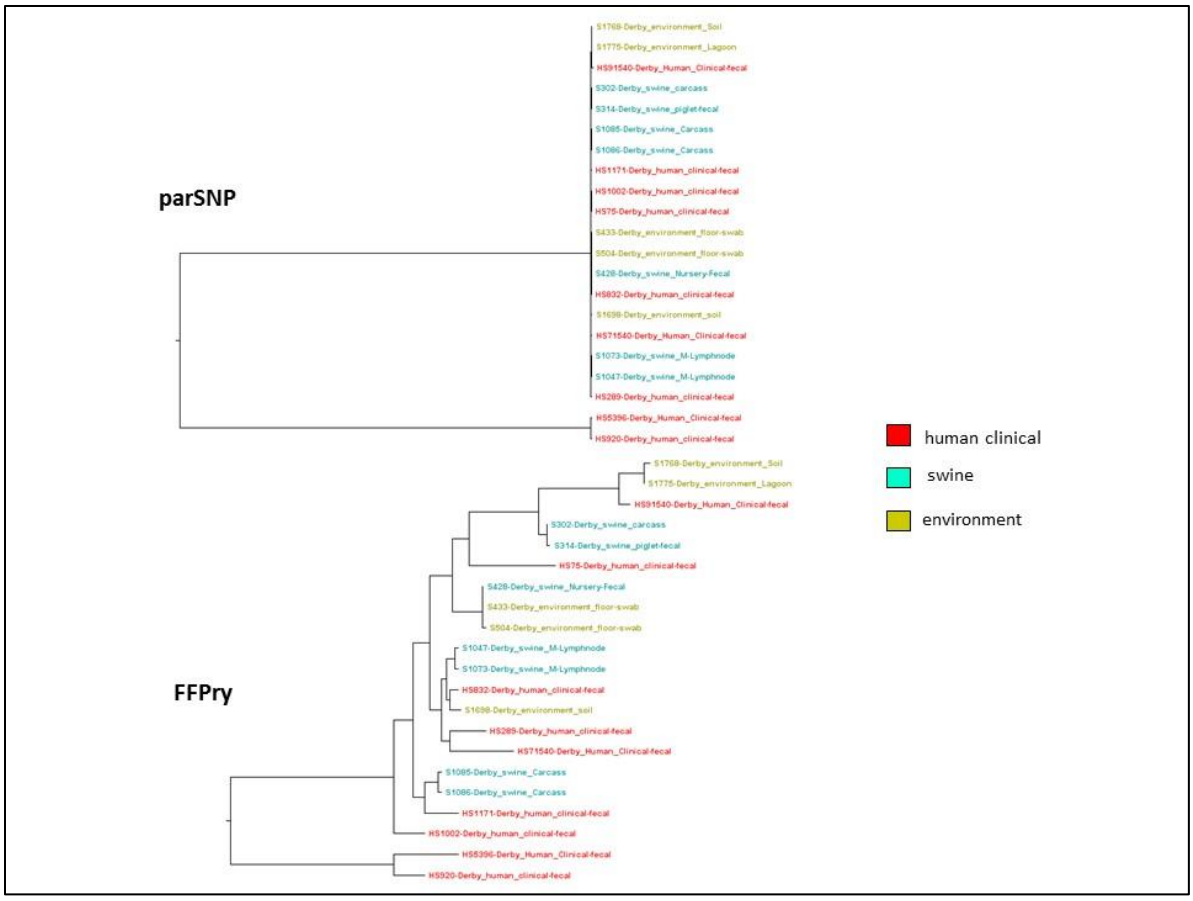
Colors indicate each source of isolate origin.

Appendix 14 Phylogenetic tree of *S.* Typhimurium isolates (*n*=39) and *S.* 4,[5],12:i:- isolates (*n*=8) recovered from human, swine, poultry, and environmental sources constructed using parSNP and FFPry analysis.



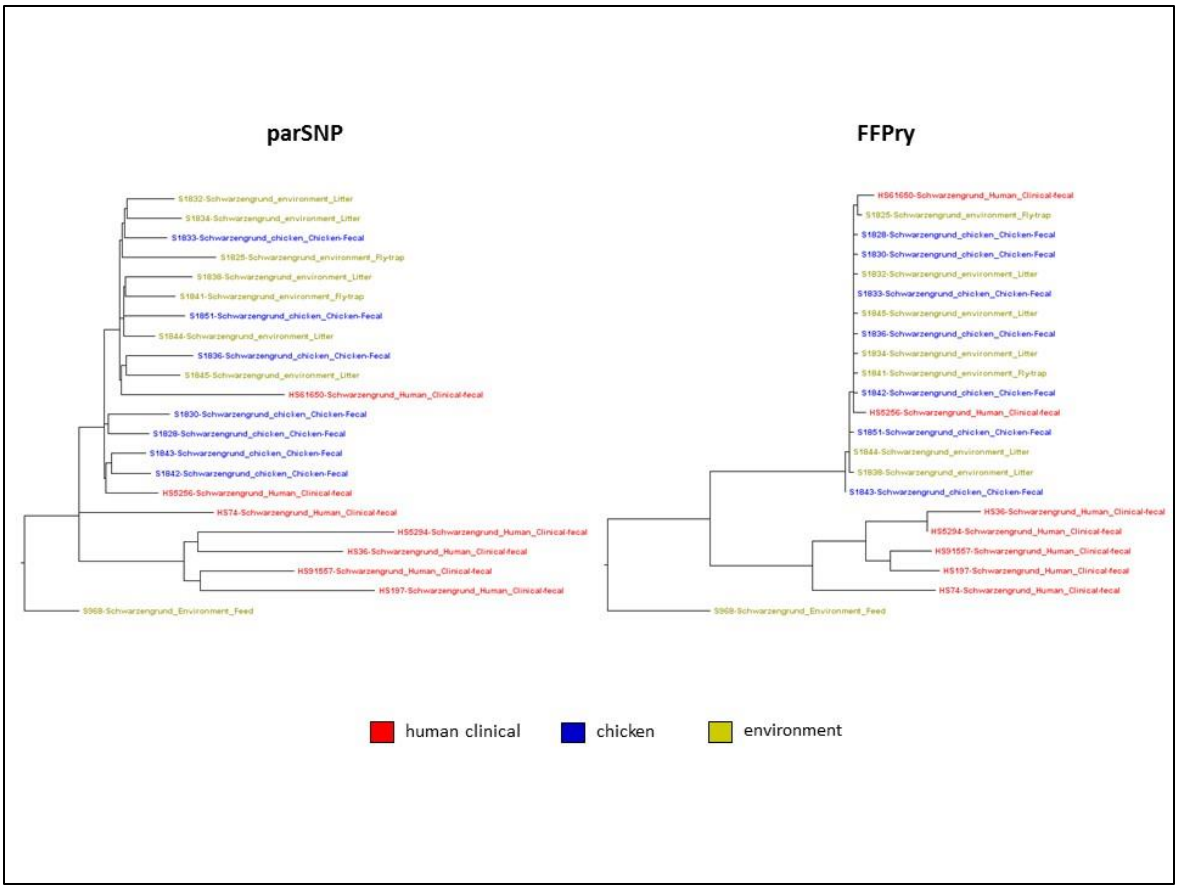
Colors indicate each source of isolate origin.

Appendix 15 Phylogenetic tree of *S.* Derby (*n*=21) recovered from human, swine, and environmental sources constructed using parSNP and FFPry analysis.



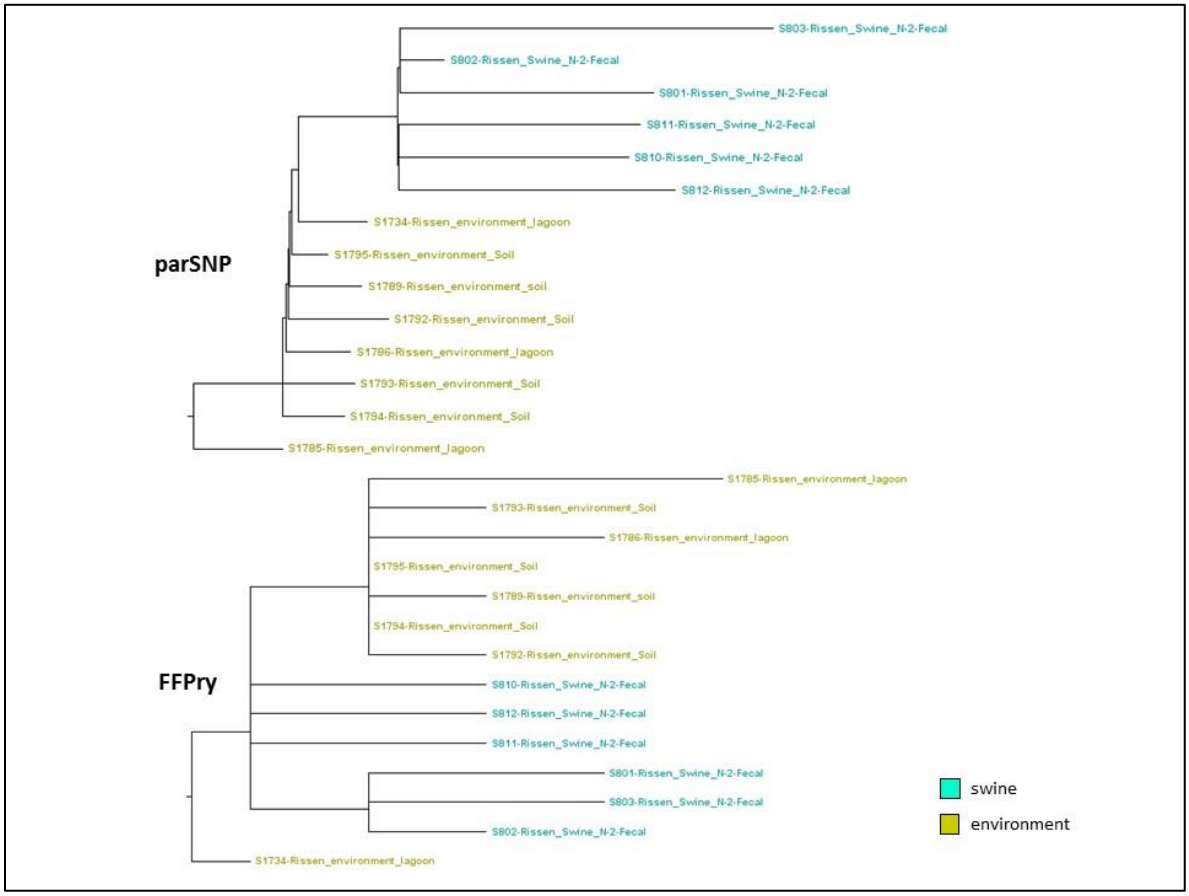
Colors indicate each source of isolate origin.

Appendix 16 Phylogenetic tree of *S.* Schwarzengrund (*n*=22) recovered from human, poultry, and environmental sources constructed using parSNP and FFPry analysis.



Colors indicate each source of isolate origin.

Appendix 17 Phylogenetic tree of *S.* Rissen (*n*=14) recovered from swine and environmental sources constructed using parSNP and FFPry analysis.



Colors indicate each source of isolate origin.