EVALUATION OF THE EVOLUTION AND CLONAL EXPANSION THROUGH PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF *SALMONELLA ENTERICA* I 4,[5],12:I:- ISOLATED FROM SWINE IN THE UNITED STATES

A Dissertation

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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December 2021

Major Subject: Biomedical Sciences

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ABSTRACT

The National Antimicrobial Resistance Monitoring System has reported a rise in multidrug-resistant *Salmonella* I 4,[5],12:i:- in both humans and animals. *Salmonella* I 4,[5],12:i:- is antigenically similar to *Salmonella* Typhimurium yet lacks the phase 2 flagellar antigen. The cause of the increased prevalence of *Salmonella* I 4,[5],12:i:- remains unknown; however, swine have been identified as an important reservoir. The aim of this study was to determine unique phenotypic and genotypic traits of *Salmonella* I 4,[5],12:i:- that have allowed this serovar to evolve and expand in swine and their environment. *Salmonella* I 4,[5],12:i:- was compared to *Salmonella* Typhimurium and other monophasic serovars to identify differences associated with monophasic expression that may affect virulence or survival.

Salmonella was isolated from head trim and cheek meat collected from healthy swine at a pork processing plant in the United States. Antimicrobial susceptibility patterns were identified by the Sensititre® system. Bacterial growth curves were determined using a BioScreen C under different antibiotic concentrations and bacterial fitness was analyzed using a 3-parameter Gompertz-model in Stata®. Motility and biofilm formation assays were used to assess swimming/swarming and biofilm production. Whole genome sequencing was performed to determine point mutations, resistance, heavy metal tolerance, and biofilm-related genes. Deletions/insertions/mutations in the *fljBA* locus were detected via alignment. The evolutionary relationship between *Salmonella* I

4,[5],12:i:- and *Salmonella* Typhimurium strains in our study, along with publicly available genomes, was evaluated via core-genome phylogenetic analyses.

All *Salmonella* I 4,[5],12:i:- isolates were multidrug-resistant and harbored the $bla_{\text{TEM-1}}$, *strA-strB, sul2,* and *tet*(B) genes encoding resistance to the common ASSuT phenotype. *Salmonella* I 4,[5],12:i:- had less fitness cost associated with *qnr*B, $bla_{\text{SHV-12}}$ and *tet* genes and had greater fitness than *Salmonella* Typhimurium under antibiotic pressure. Monophasic expression was shown to affect swimming motility but not swarming, while biofilm production was not affected but positively influenced by the presence of *sdiA*. This study is important to determining the characteristics of *Salmonella* I 4,[5],12:i:- that have led to an increased prevalence in swine for preventing salmonellosis linked to swine and pork products. Our results suggests the lack of FljB does not affect the success of *Salmonella* I 4,[5],12:i:- in swine.

DEDICATION

If I were to ask my 18-year-old self, “where do you see yourself 10 years from now?” I would not have the slightest idea. As a first-generation college student, I was unaware of all the opportunities in front of me and lacked confidence in myself. I have been blessed with meeting wonderful people who have guided me along my personal and professional journey. This dissertation is dedicated to them as I would not be where or who I am today. To my loving husband, Samuel, whose unconditional love, and support kept me going through stressful times and sleepless nights. His ray of sunshine always brought me happiness and would brighten up my toughest days. To my family, who always believed I could achieve anything I set my mind to. To my precious friends, Naomi and Maribel, who encouraged me every step of the way and were always there to lend a hand. To my undergraduate professor, Dr. Ruby Ynalvez, who saw the potential in me I did not see and introduced me to the world of research. To my advisor, Dr. Keri Norman, who was patient and understanding throughout my new and difficult journey. To my beautiful mother, who as a single mother has taught me how to be resilient and always follow my dreams. Her hard work has given me the chance to continue my education and realize my dream of becoming a Dr. “Anything is possible when you have the right people there to support you.” From the bottom of my heart, thank you.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Keri Norman, who gave me the opportunity to pursue and achieve this dream. I thank her for all of her support and guidance throughout my doctoral research. Most of all, I thank her for always seeing the best in me and giving me the confidence to believe in myself. I would also like to thank my committee member Dr. H. Morgan Scott for his support and guidance throughout the years. I thank him for sharing his immense knowledge through his challenging courses and our lab meetings. Thank you to Dr. Sara Lawhon for her support and kindness and for sharing her expertise and love for *Salmonella* and microbiology. I thank Dr. Roger B. Harvey for providing the isolates for my research project, for enlightening me with the real-world applications of our research, and for motivating me each time I went by his office by saying "this too shall pass." To my committee members, I cannot express my gratitude enough. I am fortunate to have worked alongside accomplished scientists who share a passion for research and public health. As a young scientist, it has inspired me to continue discovering and working in the field of microbiology, epidemiology, and public health.

Thank you to Dr. Michael Dickens from the Texas A&M High Performance Research Computing center as well as Pilar Donado and Oscar Gabriel Beltran from Corporación Colombiana de Investigación Agropecuaria – Agrosavia for consulting and assisting with the bioinformatics analyses.

Many thanks to my friends, colleagues, and the department faculty and staff for making my time at Texas A&M University a great experience. Great thanks to Dr. Javier Vinasco, our lab manager, for his help in developing the methodology and solving any issues that occurred during my experiments with his creative thinking. Also, thanks for the support and motivation to keep going even when my experiments failed. Thanks also go to Roberta Pugh, for managing the lab and ordering supplies. Thanks for helping graduate students decompress by hosting lab gatherings. A big shout out to all lab members and student workers for helping behind the scenes. Many thanks to Prince Agyapong and Crystobal Garza for allowing me to mentor them through the Aggie Scholars Research Program and for their eagerness to learn and help me with my many projects.

A special thanks to my dearest friends, Naomi Ohta and Maribel Leon. Thank you for always being there for me through thick and thin. No matter the distance, from Japan to Colombia, I have felt your love and support and I cannot imagine how I could have survived the doctoral program without you two. Thanks for sharing insights into being a graduate student and researcher. Above all, thank you for your beautiful friendship and for the unforgettable memories.

To my caring mother, I thank her for the love and support throughout my time as a graduate student. Thanks for listening to me talk about my research even though you knew you would not understand, but I know you tried. Thank you for showing me that with hard work and dedication one can succeed in all aspects of life. I greatly appreciate

all that you have done for me. Finally, I want to thank my beloved husband for his patience and endless love. Thank you for believing in me when I doubted myself time and time again. Thank you for being a part of the biggest chapter of my life and for being my safe place, a place where I always found love, understanding, and acceptance. I will forever treasure your love and golden heart.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of advisor and principal investigator Dr. Keri Norman of the Department of Veterinary Integrative Biosciences, committee members Dr. H. Morgan Scott and Dr. Sarah Lawhon of the Department of Veterinary Pathobiology, and committee member Dr. Roger B. Harvey of the United States Department of Agriculture – Agricultural Research Service – Food and Feed Safety Research Unit (USDA-ARS-FFSRU) in College Station, TX.

The isolate collection used in the current study along with their phenotypic characterization was provided by Dr. Roger B. Harvey of the USDA-ARS-FFSRU. Isolates were from a study conducted by Dr. Roger B. Harvey entitled "Prevalence and characterization of Salmonella from head meat and trim for ground at pork processing facilities," which was funded by the National Pork Board (grant ID:14-203). The data for section 4.8 Phylogenetic analyses of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains was provided by Pilar Donado-Godoy and bioinformatician Oscar Gabriel Beltrán of the Corporación Colombiana de Investigación Agropecuaria – Agrosavia, Centro de Investigación Tibaitatá. Part of the bioinformatics research was conducted with advanced computing resources and assistance provided by Texas A&M High Performance Research Computing. Undergraduate student research workers Prince

Agyapong (3.2.2 Bacterial fitness growth curves and 3.2.4 Biofilm assays) and Crystobal Garza (3.2.2 Bacterial fitness growth curves) assisted in performing wet lab experiments.

All other work conducted for the dissertation was completed by the student independently.

Funding Sources

Graduate study was supported by the Texas A&M University Pathways to the Doctorate Fellowship. This work was also made possible in part by the Texas A&M University – College of Veterinary Medicine (TAMU-CVM) Student Trainee Research Grant which provided funds for bacterial fitness experiments (3.2.2 Bacterial fitness growth curves). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the TAMU-CVM. All other projects were funded by start-up funds of Dr. Keri Norman.

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1. INTRODUCTION

1.1. Background

1.1.1. *Foodborne* Salmonella enterica

Non-typhoidal (non-Typhi) *Salmonella enterica* is one of the top five foodborne pathogens and one of the leading causes of foodborne illness in humans in the United States and worldwide [1, 2]. *Salmonella* is estimated to cause over 1.3 million infections annually in the United States, with nearly 26,500 hospitalizations and 420 deaths (CDC, 2020). Of the 1.3 million infections, nearly 1 million are attributed to contaminated food. In 2018, FoodNet reported the incidence of *Salmonella* infections in the United States to be 18.3 per 100,000 individuals, second only to *Campylobacter* infections [3]. Symptoms include diarrhea, fever, abdominal cramps, and sometimes vomiting [4]. Typically, salmonellosis is self-limiting, where most cases recover without treatment within 4 to 7 days. However, young children, older adults, and immunodeficient persons are at greater risk for severe infections. *Salmonella* causes mild to severe illness by invading the gastrointestinal tract; however, invasive infections sometimes can be fatal.

Salmonella is a gram-negative rod-shaped bacterium belonging to the family *Enterobacteriaceae* that can be transmitted from animals to humans directly through contact or indirectly through the food chain, resulting in zoonotic disease [5]. While bacteria as host specialists have a narrow host range – such as *Salmonella* Choleraesuis which are highly adapted to swine – host generalists like *Salmonella* Typhimurium or Enteritidis have a broad host range and can colonize or infect a variety of animals. A small

number of *Salmonella enterica* serovars are responsible for the majority of cases of human salmonellosis and typically colonize a range of animal hosts [6, 7]. Swine, bovine, and poultry each carry different strains of *Salmonella* and only certain strains cause disease in these hosts, with the remaining strains carried in a subclinical status or in a commensal relationship [8].

As a result, food animals are known to be important reservoirs for transmission of *Salmonella* to the human population. *Salmonella* can be transmitted through contact with contaminated animals, humans, or fomites. However, the most common way for *Salmonella* to be transmitted is through consumption of contaminated food or animal products. *Salmonella* is known to persist in the environments of food animal production in manure, wastewaters, and dust due to animal waste contamination which contributes to the dissemination of *Salmonella* into the food chain. Additionally, food or animal products can also become contaminated through cross-contamination, as a result of food processing procedures such as the handling of animals, processing at slaughter, or post-processing contamination from equipment or the environment. Thus, implementing proper food safety practices are important for preventing *Salmonella* infections attributed to food animals or products.

1.1.2. Monophasic Salmonella *Typhimurium:* Salmonella *I 4,[5],12:i:-*

Currently, there are more than 2,600 *Salmonella* serovars classified among the two species, *Salmonella enterica* and *Salmonella bongori*, as identified through the traditional White-Kauffman-Le Minor serotyping scheme, with the vast majority of the serovars belonging to *Salmonella enterica* [9, 10]. This method of *Salmonella* serotyping is based on the antigenic variability of O (somatic) antigens and H (flagellar) antigens that are identified via agglutination with specific antisera. The majority of *Salmonella* strains are motile, with the H antigens expressed, and are comprised of peritrichous flagella encoded by two different flagellar antigen genes, *fli*C and *flj*B, on the bacterial chromosome. These two genes are alternatively expressed to produce the flagellin proteins FliC (phase 1) and FljB (phase 2), respectively, through a regulated mechanism of the H segment, known as flagellar phase variation. Phase variation is catalyzed by site-specific recombination and reversible inversion of the *fljBA* promoter flanked by inverted repeated sequences *hix*L and *hix*R [11, 12]. Inversion of this promoter is mediated by the Hin recombinase encoded by the *hin* gene. When *fljBA* is transcribed, *flj*BA is expressed for production of FljB and FljA to inhibit *fliC* flagellin transcription. However, when the *fljBA* promoter is inverted, transcription of *fljBA* does not occur allowing transcription and expression of *fliC* for production of FliC. *Salmonella* that express both phase 1 and phase 2 flagellar antigens are considered biphasic strains, whereas, *Salmonella* which lack phase 1 or phase 2 expression are monophasic strains.

Salmonella enterica subspecies *enterica* serovar I 4,[5],12:i:- (*Salmonella* I 4,[5],12:i:-) is antigenically and genetically similar to *Salmonella enterica* subspecies

enterica serovar Typhimurium (*Salmonella* Typhimurium). However, *Salmonella* I 4,[5],12:i:- is a monophasic variant that lacks the phase 2 flagellar antigen encoded by the *flj*B gene; thus, it is referred to as monophasic *Salmonella* Typhimurium. Previous studies have found various genomic mutations and deletions in either the *flj*B gene, *flj*AB operon or surrounding genes that have resulted in the lack of phase 2 flagellar expression [13, 14]. Although less common, monophasic *Salmonella* Typhimurium strains that are serologically negative for the phase 2 flagellar antigen but are positive for the *flj*B gene are known as atypical *Salmonella* Typhimurium variants. These variants are characterized by point mutations or partial deletion of the *flj*B, *flj*A, or *hin* genes [15]. Importantly, studies have found insertion of resistance gene cassettes or mobile genetic elements within the *flj*B region harboring antimicrobial resistance (AMR) genes, heavy metal tolerance (HMT) genes, and virulence genes [16, 17].

Salmonella I 4,[5],12:i:- is now the fifth most frequently reported serovar in the United States and the third in Europe [18]. The rapid emergence of this serovar is a global health concern. Three distinct clones with varying deletion and resistance patterns have been identified since the emergence of *Salmonella* I 4,[5],12:i:-, these are: the Spanish, European, and United States clones. Analyses of genomic deletions and mutations of the phase 2 flagellar antigen region indicate multiple emergence events of this particular serovar, resulting in different lineages unique to any given geographical region [13, 19-21].

1.1.3. Antibiotic resistant Salmonella *I 4,[5],12:i:- in swine*

Food animals in the United States and Europe are administered antibiotics to prevent, control, and treat infectious disease. Consequently, resistant *Salmonella* strains can emerge and spread through meat products, fresh produce contaminated by untreated water or soil, prepared foods through contaminated surfaces, and the environment, and may cause untreatable infections in humans [22]. Unlike other host-specific serovars, *Salmonella* I 4,[5],12:i:- is similar to *Salmonella* Typhimurium in that it is not host specific. In addition to humans, *Salmonella* I 4,[5],12:i:- has been found in swine, cattle, wild-animals, and poultry [23]. Thus, the broad host range of *Salmonella* I 4,[5],12:i:- strains is a potential advantage to their relative fitness and success in colonizing and invading a host.

In recent years, the Centers for Disease Control and Prevention (CDC) have reported a rise in human foodborne illnesses related to multidrug-resistant (MDR; resistant to three or more antibiotic classes) *Salmonella* I 4,[5],12:i:-. Several foodborne outbreaks caused by *Salmonella* I 4,[5],12:i:- have been reported worldwide, including in the United States and Europe [24-27] where *Salmonella* I 4,[5],12:i:- infections are largely associated with the consumption of contaminated pork [28]. In 2015, there was a multistate outbreak in the United States of MDR *Salmonella* I 4,[5],12:i:- infections linked to pork products [25]. Other studies have also found swine to be an important reservoir for *Salmonella* I 4,[5],12:i:- [20, 27, 29-31]. However, the reasons for *Salmonella* I 4,[5],12:i:- establishing an ecological niche in swine is not fully understood. Previous studies have shown that *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium exhibit comparable ability to

persist and cause clinical disease in swine, indicating the expression of one flagellar phase does not affect the pathogenicity of monophasic *Salmonella* Typhimurium [32-34].

Furthermore, the National Antimicrobial Resistance Monitoring System (NARMS) has reported a rise in MDR *Salmonella* largely driven by an increase in *Salmonella* I 4,[5],12:i:- which includes resistance (resistance type; R-type) to ampicillin (A), streptomycin (S), sulfonamides (Su), and tetracycline (T) (ASSuT) [35, 36]. From 2014 to 2015, R-type ASSuT *Salmonella* I 4,[5],12:i:- increased from 43% to approximately 60% among human isolates. Additionally, 65% of swine cecal *Salmonella* I 4,[5],12:i:- isolates were R-type ASSuT [35]. This phenotype is commonly associated with the presence of $bla_{\text{TEM-1}}$, *strA- strB, sul*2, and *tet*(B) resistance genes, respectively [21]. Despite the accepted principle that resistance profiles aid in the selection, survival and propagation of certain bacterial strains, the link between resistance, fitness cost, and selective advantage remains poorly understood for *Salmonella* I 4,[5],12:i:-.

1.1.4. Important Salmonella *virulence factors*

Salmonella have many virulence mechanisms used to evade the host immune systems and to establish infection. Flagella are a crucial virulence factor needed for motility and chemotaxis within the host; importantly, they also take part in the induction of proinflammatory response and inhibition of apoptosis in epithelial cells [37]. Additionally, studies have shown that flagellar phase variation in biphasic *Salmonella* strains provides potential advantages in escaping detection of the immune response, adapting to new environmental niches, and evading predation [38-40]. *Salmonella* are

capable of swimming and swarming motility which allow directed movement through liquid and multi-cellular movement through semisolid surfaces, respectively [41]. Currently, there is no published research describing differences in motility between monophasic and biphasic *Salmonella* Typhimurium strains.

In addition, there are distinct adhesion factors that play a major role in *Salmonella* colonization and that are involved in biofilm formation. Biofilms are a known food safety concern as they allow bacteria to attach to human tissue, food, other biotic and abiotic surfaces and can provide resistance to antibiotics, chemical, physical and mechanical stresses [42]. Interestingly, flagella are also involved in the initial attachment of *Salmonella* to a surface and appear to be a structural component of biofilms [43]. The most important virulence genes contributing to the pathogenesis of *Salmonella* are located within the *Salmonella* pathogenicity islands (SPIs), particularly SPI-1 and SPI-2 which encode for the Type III Secretion Systems (T3SS) and secrete effectors that mediate cell invasion and survival inside host cells [44]. Other virulence factors involved in the intracellular stage of disease can be found on a virulence plasmid (pSLT) encoding the *spv* gene cluster [45, 46]. Thus, virulence factors (biofilm-associated genes) encoded within SPIs and pSLT contribute to *Salmonella* pathogenicity and disease.

In order to understand the ecological success of *Salmonella* I 4,[5],12:i:- in swine and its ability to cause infection in humans, studies are needed to investigate the differences in virulence traits and pathogenicity between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium. The loss of phase 2 flagellar expression in *Salmonella* I

4,[5],12:i:- isolated from swine may be attributed to different selection pressures in swine production. Virulence factors involved in motility and biofilm formation, AMR, HMT, and bacterial fitness, may play an important role in the selective and competitive advantages of *Salmonella* I 4,[5],12:i:- in swine.

1.2. Study objectives

The phenotypic and genotypic characteristics of *Salmonella* I 4,[5],12:i:- that have led to the successful evolution and expansion of this serovar, particularly in swine and their environment, are still poorly understood. The overall objective of this study is to identify phenotypic and genotypic traits that may have permitted *Salmonella* I 4,[5],12:i:- to emerge and establish an ecological niche in swine production and pork processing, leading to substantially increased attribution of foodborne illness. We have a unique collection of *Salmonella* I 4,[5],12:i:- isolates and other important serovars, previously isolated from head trim and cheek meat of market hogs in the United States and collected over a one-year timeframe. There are three aims proposed to achieve the overall objective of this study:

1.2.1. Aim 1: Genotypic analysis

Determine the genotypic characteristics of *Salmonella* I 4,[5],12:i:- likely to be associated with a selective and competitive advantage over other strains. For this aim, whole-genome sequencing (WGS) was used to determine the multilocus sequence type (MLST) of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains, as well as the

presence of antimicrobial resistance genes, plasmids, heavy metal resistance genes and biofilm-associated genes.

1.2.2. Aim 2: Phenotypic analysis

Determine the phenotypic traits of *Salmonella* I 4,[5],12:i:- that may be related to increased fitness in swine and their environment compared to other serovars. To achieve this aim, antimicrobial susceptibility testing, bacterial fitness growth curves, motility assays, and biofilm assays was performed on *Salmonella* I 4,[5],12:i:-, *Salmonella* Typhimurium, and other monophasic *Salmonella* strains. Differences among their antimicrobial resistance profiles, fitness costs of associated resistance genes, swimming and swarming abilities, and biofilm formation was assessed.

1.2.3. Aim 3: Evolutionary Analysis

Evaluate the evolutionary relationship and genetic relatedness between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from swine from the current study, as well as the genetic relatedness of *Salmonella* I 4,[5],12:i:- from publicly available genomes. For this, deletions and mutations around the *fljBA* locus were assessed by mapping our *Salmonella* I 4,[5],12:i:- strains to the *Salmonella* Typhimurium str. LT2 reference genome (NC_003197). In addition, SNP-based phylogenetic trees were developed using the *Salmonella* Enteritidis str. 18569 (GCA_000335875.2) reference genome and *Salmonella* I 4,[5],12:i:- USDA15WA-1 outbreak strain (NCBI accession number CP040686) was included in the analyses.

1.3. Significance

Salmonella I 4,[5],12:i:- has been increasing in prevalence and incidence in the last several years. In the United States, *Salmonella* I 4,[5],12:i:- is the fifth most frequently reported serovar implicated in human infections and has been increasingly isolated from swine and pork products. In this research study, we investigated potential traits unique to *Salmonella* I 4,[5],12:i:-, that may allow it to have a competitive fitness advantage, resulting in increased incidence in humans and increased prevalence in swine. The *Salmonella* isolates included in this study are representative of strains found in commercial, healthy slaughter-age swine in the United States that have the potential to enter the food chain and cause salmonellosis in humans. The *Salmonella* I 4,[5],12:i:- isolates in our collection are representative of several phenotypic antimicrobial resistance profiles, each of which has been reported in outbreaks in the United States and abroad. Furthermore, the *Salmonella* I 4,[5],12:i:- resistance profiles explored in this study are of great importance, given that infections caused by antibiotic-resistant *Salmonella* are more difficult to treat. Importantly, the identification of competitive advantages could help in making decisions to prevent future *Salmonella* I 4,[5],12:i:- infections associated with pork products; that is, the information gained from this study may lead to potential mitigation strategies for controlling this serovar in the swine industry.

2. LITERATURE REVIEW

2.1. Salmonella *description and background*

2.1.1. Taxonomy

Salmonella, which is closely related to the genus *Escherichia*, is composed of Gram-negative bacilli belonging to the *Enterobacteriaceae* family. *Salmonella* are flagellated and facultative intracellular anaerobes consisting of two species: *Salmonella enterica* and *Salmonella bongori* (also known as subspecies V). *Salmonella enterica* is further subdivided into six subspecies: *enterica* (I), *salamae* (II), *arizonae (*IIIa), *diarizonae* (IIIb), *houtenae* (IV), and indica (VI) (Figure 2-1). Within *Salmonella enterica* subspecies I, there are serovars grouped into agents of typhoid and paratyphoid fevers (typhoidal) or agents of gastroenteritis and extra-intestinal illnesses (non-typhoidal) [47, 48]. All *Salmonella* serovars are classified into serovars based on the somatic (O) surface antigen, flagellar (H) antigens, and occasionally capsular (Vi) antigens via traditional serotyping with the White-Kauffmann-Le Minor scheme [9]. There are over 2,600 serovars within the two species, most belonging to the species *Salmonella enterica* while *Salmonella bongori* has only 22 serovars. Additionally, subspecies I comprises more than half of the serovars compared to the other subspecies combined [10].

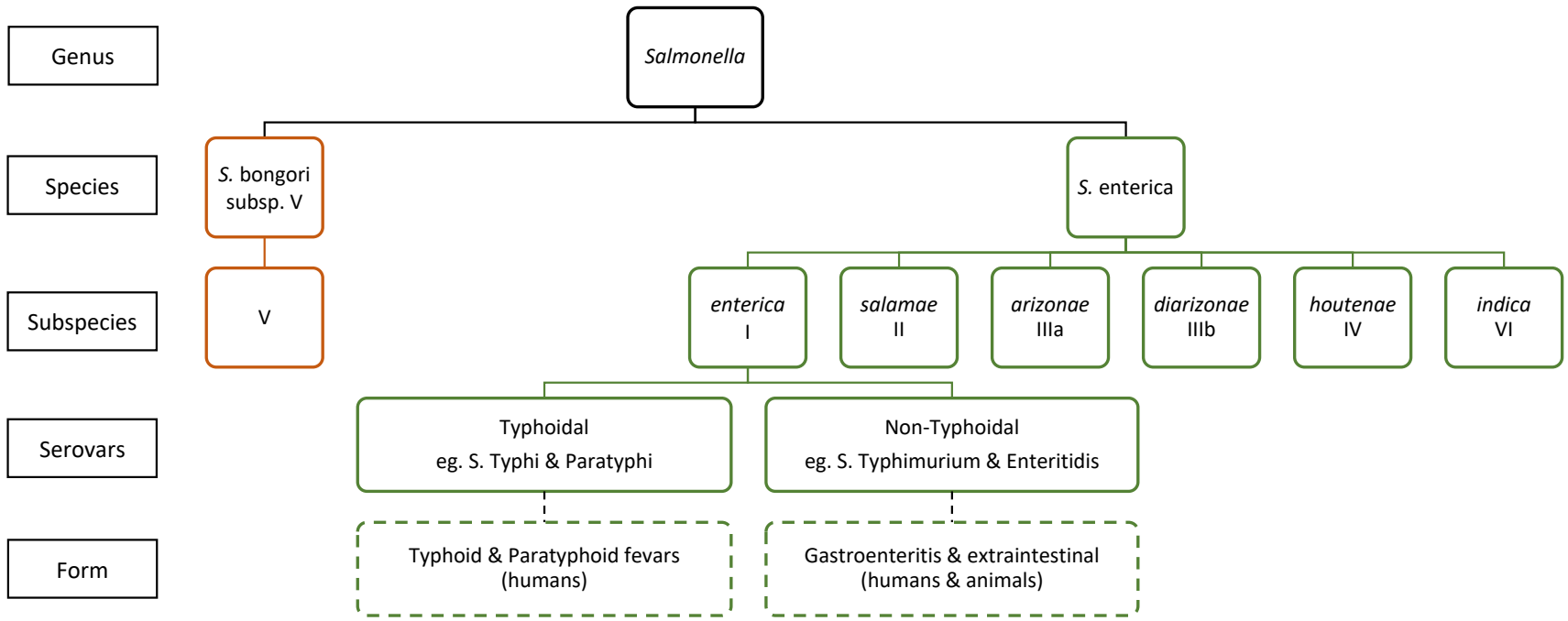


Figure 2-1 Classification of *Salmonella* species and subspecies adaptation from Scallan et al.

2.1.2. Biophysiological properties

The length and diameter of *Salmonella* ranges from 2 to 5 μm and 0.7 to 1.5 μm, respectively. Approximately 6 to 8 peritrichous flagella are distributed over the entire cell surface. The flagella are used for motility as well as facilitation of adhesion and biofilm formation. *Salmonella* serovars grow at temperatures between 5 to 47°C and pH levels of 4 to 9; however, the optimum temperature is 35 to 37°C and optimum pH is 6.5 to 7.5. *Salmonella* require high water activity (a_w) in their surrounding environment [49]. Biochemical characteristics of the genus *Salmonella* include the ability to ferment glucose, mannitol, and sorbitol to produce acid and gas or simply acid. Additionally, *Salmonella* are catalase and hydrogen sulfide positive as well as lactose, sucrose, and oxidase negative. Non-typhoidal *Salmonella* are also lysine decarboxylase positive [50].

2.1.3. Host specificity

From a clinical perspective, serovars of *Salmonella enterica* subspecies I (henceforth, referred to as *Salmonella*) can be divided into three host specificities on the basis of host-prevalence: host-restricted, host-adapted, and host-generalist. Host-restricted serovars are associated with enteric fever and a systemic invasive disease, in a single host. This includes *Salmonella* Typhi and *Salmonella* Paratyphi A, B, and C which are found only in humans and cause forms of typhoid fever. Other serovars, such as non-typhoidal *Salmonella* (NTS), may be host-adapted and cause infection mainly in a single host but are capable of infecting other species or are host-generalists causing gastroenteritis in a broad-range of host species. NTS primarily consists of generalists that can infect multiple

species including humans, pigs, cattle, birds, and reptiles. Examples of host-adapted serovars include *Salmonella* Choleraesuis and *Salmonella* Dublin which are specifically adapted to pigs and cattle, respectively. Whereas, *Salmonella* Enteritidis, *Salmonella* Newport, and *Salmonella* Typhimurium are host-generalists that are capable of colonizing and infecting both humans and animals [7]. These host-generalist serovars are the most common serovars responsible for salmonellosis in humans and are the top three frequently reported serovars in the United States by the Centers for Disease Control and Prevention (CDC) [18].

Non-typhoidal *Salmonella* is one of the leading bacterial pathogens responsible for foodborne illnesses, and infections are commonly associated with consumption of contaminated food such as pork, beef, or poultry [51, 52]. Food animals are known to be important reservoirs of NTS serovars and often are asymptomatic carriers. Consequently, there is a risk of *Salmonella* entering the food chain undetected and causing disease in humans. The ability of *Salmonella* to infect different hosts represents their ability to replicate and survive in diverse environmental niches [7]. For example, although *Salmonella* Enteritidis can infect different hosts, several *Salmonella* Enteritidis outbreaks have been linked to the consumption of raw or undercooked eggs or egg products [23, 53]. From 1968 to 2011, the National Veterinary Services Laboratories (NVSL) of the United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) reported a large percentage of *Salmonella* Enteritidis isolated primarily from chickens with clinical and non-clinical signs of salmonellosis [54]. It has been hypothesized that

Salmonella Enteritidis has filled the ecological niche following eradication of host-restricted *Salmonella* Gallinarum [55, 56]. In general, host specificity is a major driver of the evolution of adaptation and virulence to certain hosts. There are many factors involved in the ability of *Salmonella* to infect different hosts and the fitness of *Salmonella* varies from host to host [47, 57]. To understand the success of a *Salmonella* serovar in a particular ecological niche, it is important to investigate their unique characteristics of pathogenicity and survival.

Salmonella I 4,[5],12:i:-, the monophasic variant of *Salmonella* Typhimurium, is also a host-generalist which can colonize or infect a broad-range of host species. However, in addition to the increase prevalence of *Salmonella* I 4,[5],12:i:- in swine, several *Salmonella* I 4,[5],12:i:- foodborne outbreaks have been associated with the consumption of contaminated pork and pork products; thus, suggesting *Salmonella* I 4,[5],12:i:- has established an ecological niche in swine, making swine an important reservoir. It remains unknown if the lack of the phase 2 flagellar antigen affects the pathogenicity of *Salmonella* I 4,[5],12:i:-. Therefore, in our study we will investigate the unique characteristics of *Salmonella* I 4,[5],12:i:- isolated from swine at slaughter to understand the success of this monophasic serovar compared to biphasic *Salmonella* Typhimurium.

2.1.4. Clinical manifestation of salmonellosis

NTS *Salmonella* infection remains a major public health concern worldwide as salmonellosis causes significant morbidity and mortality. In the United States alone, there are more 1.3 million infections of salmonellosis every year with almost 1 million related

to sources of contaminated food [58]. NTS infections in humans are characterized by gastroenteritis and can occur through the ingestion of contaminated food or water sources, where the infective dose is between $\leq 10^1$ to 10^9 colony forming units (CFU). Typically, symptoms such as diarrhea, fever, nausea, abdominal cramps, and occasionally vomiting, occur after 12 to 72 hours. In immunocompetent individuals, symptoms are usually self-limiting and last less than 10 days. Infants, young children, older adults, and immunodeficient individuals are at greater risk and can develop more severe symptoms which can lead to bacteremia or life-threatening complications. Disease outcomes of infections vary by serovar and susceptibility of the individual [1, 4].

2.1.5. Antimicrobial resistance

Antimicrobial resistance (AMR) is a global phenomenon resulting in the emergence of bacterial pathogens with resistance to critically important antimicrobials for both human and animal health. The Centers for Disease Control and Prevention (CDC) classified drug-resistant NTS as a serious public health threat [59]. In the United States, approximately 212,000 NTS drug-resistant infections and 70 deaths occur annually [60]. In self-limiting NTS infections, treatment of salmonellosis often includes oral or intravenous rehydration of fluids and electrolytes. However, antibiotics such as azithromycin, ciprofloxacin and ceftriaxone are used in severe and invasive *Salmonella* infections [59]. Ciprofloxacin is a fluoroquinolone antibiotic used for treatment in adults. Ceftriaxone is a third-generation cephalosporin antibiotic often used for treatment of *Salmonella* infections in the higher risk population such as infants, children, women who

are pregnant and immunosuppressed individuals. Ceftriaxone is the primary choice because ciprofloxacin, or fluoroquinolones in general, are associated with adverse effects including genotoxicity, neurotoxicity, and musculoskeletal toxicity [7-11]. Thus, fluoroquinolones are not recommended and reserved only when there is no safe alternative. Ciprofloxacin and ceftriaxone are considered highest priority critically important antimicrobials by the World Health Organization (WHO) [60]. Consequently, there has been an increase in resistance to these antibiotics in NTS isolates over the past few years which made infections related to these isolates difficult to treat and control. Food animals such as poultry, cattle, and swine have been linked as common sources of AMR NTS leading to drug-resistant *Salmonella* infections. Antibiotics are commonly used in food animals to prevent, treat, and control infections resulting in the emergence and spread of AMR *Salmonella* [61, 62]. AMR NTS have been associated with a large number of foodborne outbreaks in the United States [5, 23]. Thus, AMR in foodborne NTS is a major concern for food safety and public health.

2.2. Emergence and epidemiology of Salmonella I *4,[5],12:i:-*

2.2.1. Salmonella *I 4,[5],12:i:- human salmonellosis cases*

Over the past few years, *Salmonella* I 4,[5],12:i:- has increased in incidence in humans. *Salmonella* I 4,[5],12:i:- has become one of the most common serotypes worldwide because of its rapid emergence and spread. In the United States, the rate of reported *Salmonella* I 4,[5],12:i:- isolated from humans has drastically increased since 1996 and it is now the fifth most common serovar [63]. While *Salmonella* Typhimurium

is the third most common serovar in the United States, the rate of reported *Salmonella* Typhimurium isolated from humans has remained stable since the late 1980s [64]. Furthermore, the 2015 National Antimicrobial Resistance Monitoring System (NARMS) Integrated Report noted a decline in *Salmonella* Typhimurium human isolates resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (R-type ACSSuT) which resulted in an overall decline in MDR *Salmonella* from 1996 to 2008. From 2008 to 2014, the overall multidrug resistance among *Salmonella* human isolates remained constant; however, in 2015, there was a slight increase driven by R-type ASSuT (resistance to ampicillin, streptomycin, sulfonamides, and tetracycline) *Salmonella* I 4,[5],12:i:- [35]. In Europe, *Salmonella* I 4,[5],12:i:- is the third most common serovar behind *Salmonella* Typhimurium. Human salmonellosis cases attributed to *Salmonella* I 4,[5],12:i:- have remained constant from 2014 to 2016 at approximately 8.0% in the U.S. During the same years, *Salmonella* Typhimurium has decreased significantly from 17.4% to 13.4%. Interestingly, the number of *Salmonella* Typhimurium isolated from human salmonellosis cases across the world have decreased while its monophasic variant *Salmonella* I 4,[5],12:i:- has increased. We can hypothesize that the ecological niche of *Salmonella* Typhimurium is being replaced or filled by the emergence of its monophasic variant, *Salmonella* I 4,[5],12:i:-.

2.2.2. Worldwide Salmonella *I 4,[5],12:i:- Outbreaks*

In the United States, there have been a handful of salmonellosis outbreaks caused by *Salmonella* I 4,[5],12:i:-. The most recent *Salmonella* I 4,[5],12:i:- multistate outbreak

occurred in 2019; 154 cases were reported from 34 states and linked to contact with pig ear dog treats [65]. Additional multistate outbreaks have been associated with frozen rodents used for reptile feed (2010), consumption of frozen poultry pot pies (2007), alfalfa sprouts (2010), pork (2015), and chicken (2018) [23]. In 2015, the *Salmonella* I 4,[5],12:i:- outbreak that infected 188 people in five states was specifically associated with consumption of whole roasted hogs. Ten *Salmonella* I 4,[5],12:i:- clinical isolates tested for antibiotic susceptibility from this outbreak were classified as MDR with the R-type ASSuT [66]. Traceback investigations based on the source of pork meat, revealed Kapowsin Meats as the common pork supplier. When this Washington slaughter and processing establishment resumed in 2016, a smaller outbreak of 15 MDR *Salmonella* I 4,[5],12:i:- infections linked to whole roasted pigs occurred again. Furthermore, *Salmonella* I 4,[5],12:i:- isolated from swine carcass and environmental samples from the establishment were closely related to clinical isolates from the previous outbreaks [67]. *Salmonella* I 4,[5],12:i:-, like *Salmonella* Typhimurium, is a host-generalist which can colonize or infect a broad-range of host species which could lead to *Salmonella* infections in humans through food products, contact with animals, or the environment. Additionally, the MDR pattern of *Salmonella* I 4,[5],12:i:- is a cause for concern infections may be difficult to treat.

Additionally, *Salmonella* I 4,[5],12:i:- has been reported as the cause of many outbreaks in Europe. In Luxembourg, there were two large *Salmonella* I 4,[5],12:i:- R-type ASSuT outbreaks (both 2006) linked to the consumption of contaminated pork [29].

Additionally, Italy had an outbreak linked to salami in 2010 and *Salmonella* I 4,[5],12:i:- R-type ASSuT was isolated from human cases. For two years following the outbreak, a Regional Surveillance Program was conducted and R-type ASSuT *Salmonella* I 4,[5],12:i:- were isolated from samples of salami collected from the producers involved in the outbreak [68]. Outbreaks in France have been associated with consumption of imported beef (2010) and dried pork sausage (2011) [26, 69] and in Spain outbreaks have been associated with consumption of dried pork sausage (2011) and roasted pork (2016) [70, 71]. Both in France and Spain, R-type ASSuT *Salmonella* I 4,[5],12:i:- strains were isolated from human cases and the associated food product. A *Salmonella* I 4,[5],12:i:- outbreak also occurred in Denmark (2018) linked to the consumption of raw pork sausage [24]. In the past two decades, *Salmonella* I 4,[5],12:i:- has rapidly emerged in several countries and has become one of the most common serovars isolated from humans and animals, particularly from swine.

In summary, *Salmonella* I 4,[5],12:i:- has been responsible for a significant number of human salmonellosis cases and foodborne outbreaks worldwide. Most importantly, foodborne outbreaks have been largely associated with the consumption of contaminated pork and pork products. These observations demonstrate the ecological success of *Salmonella* I 4,[5],12:i:- in swine and swine production. Therefore, swine have been identified as an important reservoir for *Salmonella* I 4,[5],12:i:-.

2.2.3. Relationship to biphasic Salmonella *Typhimurium*

Salmonella I 4,[5],12:i:- is the monophasic variant of *Salmonella* Typhimurium, as it is antigenically and genetically similar to *Salmonella* Typhimurium. There have been several studies that have confirmed the relatedness of these two serovars [15, 21, 27]. De la Torre *et al.* discovered over 78% similarity between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates of swine origin, including their phage type (U302), AMR profile (R-type ACSSuT), pulsed-field gel electrophoresis type (PFGE) and plasmid profiles, suggesting that the *Salmonella* I 4,[5],12:i:- U302 originated from *Salmonella* Typhimurium U302 [27]. Additionally, Lucarelli *et al.* also observed PFGE and AMR profile (R-type ASSuT) similarities among *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium human isolates from Denmark, Italy, and the United Kingdom [21]. In Spain, human, pork, and pork product *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates also shared the same phage type (U302) and AMR pattern (R-type ACSSuGTSxT; ampicillin, chloramphenicol, streptomycin, sulfonamides, gentamycin, tetracycline, and trimethoprim/sulfamethoxazole) [15].

Aside from PFGE and AMR profiles, *Salmonella* I 4,[5],12:i:- strains have also been found to have *Salmonella* Typhimurium-specific sequences. Hopkins *et al.* detected the malic acid dehydrogenase gene (*mdh*) specific to *Salmonella* Typhimurium in all *Salmonella* I 4,[5],12:i:- isolated from humans, pigs, and pork products in several European countries. Ninety-seven percent of these *Salmonella* I 4,[5],12:i:- isolates were phage types DT193 and DT120 with R-type ASSuT indicating these strains were not related to the U302 *Salmonella* I 4,[5],12:i:- strains that emerged from Spain [72].

Although the majority of Spanish *Salmonella* I 4,[5],12:i:- isolates are phage type U302, other phage types typical of *Salmonella* Typhimurium, such as DT104, DT208, and DT193, have also been found in Spain and other European countries [19, 27, 73]. Thus, there are a diversity of phage types of *Salmonella* I 4,[5],12:i:- linked to *Salmonella* Typhimurium. In our study, the multilocus sequence type (MLST) and AMR profiles of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolated from swine in the United States will be determined to evaluate the relationship between the two serovars using whole-genome sequencing (WGS). Furthermore, WGS will allow us to evaluate the relationship between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium at the nucleotide level and observe differences within the phase 2 flagellar antigen region.

WGS and phylogenetic analyses of bacterial genomes have become useful tools in the characterization and comparison of bacterial strains. Through WGS, evolutionary relationships and relatedness of *Salmonella* serovars may be determined by examining similarities and differences between isolates on a genomic level. Petrovska *et al.* performed phylogenetic analyses on *Salmonella* I 4,[5],12:i:- isolated from the United Kingdom and Italy in 1993 to 2010 and found the majority (~79%) formed a single clade different from *Salmonella* I 4,[5],12:i:- clones (2005-2010) in the United States and Spain. Interestingly, older *Salmonella* I 4,[5],12:i:- isolates (~21%) were in other clades with *Salmonella* Typhimurium isolated before 2005 [14]. Many studies have shown *Salmonella* I 4,[5],12:i:- to have identical or similar genotypic profiles as *Salmonella* Typhimurium

through MLST, PFGE, AMR profiles, plasmid profiles, and IS200 typing establishing the relatedness of *Salmonella* I 4,[5],12:i:- to *Salmonella* Typhimurium.

The characterization of *Salmonella* I 4,[5],12:i:- isolates from several countries supports the hypothesis that *Salmonella* I 4,[5],12:i:- has repeatedly emerged from *Salmonella* Typhimurium followed by clonal expansion, particularly in swine. WGS comparisons of *Salmonella* I 4,[5],12:i:- in our study will be performed to determine the genetic relatedness among *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from swine through phylogenetic analyses. The phylogenetic analyses will aid in determining whether the *Salmonella* I 4,[5],12:i:- isolated from swine in the United States form a single epidemic clade or are related to previously circulating biphasic and monophasic *Salmonella* Typhimurium strains in the United States or other countries. The genotypic variation affecting flagellar expression of *Salmonella* I 4,[5],12:i:-, as well as AMR genes, heavy metal tolerance genes, and virulence factors among isolates will be compared to help explain the emergence and expansion of *Salmonella* I 4,[5],12:i:- in swine.

Furthermore, we will explore the presence of various mobile genetic elements inserted within the *fljAB* region of *Salmonella* I 4,[5],12:i:- isolates and encoding antimicrobial resistance genes and/or heavy metal tolerance genes through WGS. Resistance to antibiotics and heavy metals provides a competitive advantage for this serovar, particularly in the swine industry where antibiotic and heavy metal use provide selection pressures [13, 17, 74-77]. Several studies have found the integration of mobile

genetic elements or regions containing MDR genes and heavy metal tolerance genes within the phase 2 flagellar region resulting in the monophasic expression of *Salmonella* I 4,[5],12:i:- [17, 36, 78, 79]. Additionally, differences have been shown in the *fljAB* region of *Salmonella* I 4,[5],12:i:- isolates from geographically distinct regions. It has been well established that *Salmonella* I 4,[5],12:i:- is a monophasic variant of *Salmonella* Typhimurium and has repeatedly emerged worldwide with swine identified as an important reservoir.

2.2.4. Prevalent clones: Spanish, European, U.S.

Since the emergence of *Salmonella* I 4,[5],12:i:-, three prevalent clones with various deletion and resistance profiles have been identified in distinct international geographic regions; the Spanish, European, and United States clones (Table 2-1). The Spanish clone was first detected by the Spanish National *Salmonella* Reference Laboratory in 1997. The Spanish clone was identified as a monophasic variant of *Salmonella* Typhimurium by PCR amplification of the Typhimurium-specific 1000 base pairs (bp) *fliB-fliA* fragment containing the *IS200* element and 162 bp U302-specific phage type region. Garaizar et al. observed a 16-gene deletion in the Spanish clone, including deletion of the *fljAB* operon (*fljA, fljB,* and *hin* genes) and the *iroB* gene (encoding for a glycosyl transferase) [80]. Additionally, the Spanish clone is MDR with resistance to the following antimicrobials: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), tetracycline (T), gentamicin (G), and trimethoprim-sulfamethoxazole (SxT) (ACSSuT-GSxT R-type) [15]. Guerra et al. found the Spanish clone harbored the

bla$_{\text{TEM-1}}$, *cmlA1*, *aaA2*, *sul1*, *tet*(A), *aac(3)-IV*, and *dfrA12* genes encoding for ACSSuT-GSxT resistance, respectively. Furthermore, Guerra et al. detected a 1,900 bp class 1 integron harboring the *dfrA12* and *aaA2* resistance gene cassettes and 120 to 140 kb plasmids harboring the *bla*$_{\text{TEM-1}}$, *aac(3)-IV*, *cmlA1*, *sul1* and *tet*(A) genes [81]. The class 1 integron resistance cassettes and plasmids carrying the AMR genes have integrated into the flagellar phase region resulting in the monophasic expression and multidrug-resistance of the *Salmonella* I 4,[5],12:i:- Spanish clone [81]. The Spanish clone has been predominantly found in Spain; however, has also been observed in Italy, Germany, and France [82]. The AMR genotypic profiles and deletion profiles of *Salmonella* I 4,[5],12:i:- isolates in our study will be determined and used to identify mobile genetic elements within the flagellar phase region causing monophasic expression.

In 2010, a study performed by Hopkins et al. revealed the European clone as a new pandemic *Salmonella* I 4,[5],12:i:- strain that had emerged in the early 2000s across different European countries (e.g. England and Wales, France, Germany, Italy, Poland, Spain, and the Netherlands) [72]. All 122 *Salmonella* I 4,[5],12:i:- isolates from the study were confirmed as monophasic variants of *Salmonella* Typhimurium through PCR. The Typhimurium-specific *mdh* gene was present in all strains and the *fljB* gene was absent. Furthermore, the majority (97%) of *Salmonella* I 4,[5],12:i:- isolates were identified as phage type DT193 followed by DT120, indicating the isolates were not related to the Spanish clone [72]. Additionally, previous studies, including the Hopkins et al. study, have found the European clone to be MDR to ampicillin (A), streptomycin (S), sulfonamides

(Su), and tetracycline (T) (R-type ASSuT) which is encoded by *bla*$_{\text{TEM-1}}$, *str*A-*str*B*, sul2* and *tet*(B) genes, respectively [21, 72, 79]. This genotypic profile is different than the previously discussed Spanish clone. Lucarelli et al. discovered *Salmonella* I 4,[5],12:i:- isolates (from Italy, Denmark, and the UK) were negative for class 1 integrons via PCR and determined the localization of the resistance genes on the bacterial chromosome via failure of horizontal gene transfer [21]. This chromosomal genomic region was later sequenced (three isolates from Italy) in a second study by Lucarelli et al. and found to contain two highly conserved adjacent resistance regions (RR1 and RR2) which were both surrounded by insertion sequence 26 (IS26) elements. RR1 harbored *bla*$_{\text{TEM-1}}$, *str*A-*str*B*,* and *sul2* resistance genes while RR2 harbored the *tet*(B) resistance gene; both showed 99% sequence identity with a region of the pO111_1 plasmid harbored by an *Escherichia coli* (*E. coli*) strain. Furthermore, the IS26 insertion resulted in a deletion of the *fljAB* operon while *iroB* remained conserved unlike the Spanish clone [83]. Another study, Boland et al., also revealed similar resistance regions (RR1 and RR2) in Belgium *Salmonella* I 4,[5],12:i:- isolates [84]. On the other hand, a study conducted by Garcia et al. showed 122 *Salmonella* I 4,[5],12:i:- strains that were identified as DT193 and ASSuT R-type strain (from Germany, Italy, and Switzerland) did not harbor RR1 or RR2. Rather the sequencing of the *Salmonella* I 4,[5],12:i:- isolates showed a different resistance region (RR3) integrated in the *fljA-fljB* operon location. The 28 kbp RR3 harbored *bla*$_{\text{TEM-1}}$, *str*A-*str*B*, sul2*, and *tet*(B) resistance genes, transposons flanked by IS26, plasmid replication genes, and open-reading frames (orfs) characteristically located on IncH1 plasmids [79]. The differences among the *Salmonella* I 4,[5],12:i:- Spanish and European clone are the

R-type, MLST and the genomic insertions/deletions in the flagellar phase region (Table 2-1) and the types of mobile genetic elements present and genotypic AMR (Table 2-2). Additionally, the AMR genes encoding resistance to sulfonamides and tetracycline differ among the two clones where the Spanish clone harbors *sul*1 and *tet*(A), while the European clone harbors *sul*2 and *tet*(B). In both cases, the integration of genetic elements or regions replacing the genes are responsible for the expression of the phase 2 flagellar antigen gene indicates stabilization of new genetic material into the chromosome of *Salmonella* Typhimurium resulting in monophasic expression.

Lastly, the U.S. clone is a pan-susceptible *Salmonella* I 4,[5],12:i:- strain. Zamperini et al. identified smaller deletions and mutations in the *fljAB* operon compared to the extensive deletion reported by Gariazar et al. in the Spanish clone. Specifically, 89% of U.S. *Salmonella* I 4,[5],12:i:- isolates had partial or complete deletions of the *fljB* gene while 11% retained the *fljB* with small deletions or point mutations. Although the deletions in the *fljB* locus differed, the *hin* and *iroB* genes were preserved in all isolates (Table 2-1) [85]. Though MDR clones are rare in the United States, R-type ASSuT *Salmonella* I 4,[5],12:i:- strains isolated from swine (by the Minnesota Veterinary Diagnostic Laboratory) in the U.S. Midwest have been identified by Elnekave et al. as an emerging MDR clade that was first reported in Europe. Elnekave et al. also found a proportion of the U.S. *Salmonella* I 4,[5],12:i:- isolates from swine resistant to enrofloxacin and/or ceftiofur [78]. Enrofloxacin resistant isolates harbored the plasmid-mediated quinolone resistance (PMQR) genes (e.g. *qnr*B19, *qnr*B2, or *qnr*S1) and/or point mutations in *parC*

and *gyrA*; most harbored *qnr*B19 followed by *qnr*B2 and *qnr*S1. Ceftiofur resistant isolates harbored plasmid-mediated *bla*$_{\text{CMY-2}}$ or *bla*$_{\text{SHV-12}}$. A phylogenetic analysis conducted by Elnekave et al. demonstrated two predominant *Salmonella* I 4,[5],12:i:- clades among isolates from different sources (e.g. livestock and humans) and geographic locations (e.g. U.S. and Europe): pan-susceptible ST19 *Salmonella* I 4,[5],12:i:- and R-type ASSuT ST34 *Salmonella* I 4,[5],12:i:- [78]. Both clades had similar proportions of U.S. clones (~80%) and European clones (~20%). The majority of *Salmonella* I 4,[5],12:i:- isolates in the pan-susceptible ST19 clade were isolated before 2014 while the majority of *Salmonella* I 4,[5],12:i:- isolates isolated after are in the R-type ASSuT ST34 clade. Performing phylogenetic analyses of *Salmonella* I 4,[5],12:i:- isolates from our study along with other *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium found in the National Center for Biotechnology Information (NCBI) database from different sources and locations will allow us to investigate the genetic relationship among strains and the potential origin of our *Salmonella* I 4,[5],12:i:- isolates.

There have been distinct *Salmonella* I 4,[5],12:i:- strains recovered internationally showing differences in phage types, PFGE profiles, resistance profiles, MLST, and genomic mutations and deletions of the phase 2 flagellar antigen region. *Salmonella* I 4,[5],12:i:- have high genetic heterogeneity and differences in the *fljAB* region with the same phenotypic and genotypic resistance indicating the evolution of *Salmonella* I 4,[5],12:i:- from *Salmonella* Typhimurium has occurred through multiple independent emergence events. The heterogeneity may be attributed to the low GC-content around the

fljAB region, which is known to be less stable than high GC-content regions of the genome [86]. The low GC-content allows for integration of plasmids, IS elements, and transposons [13, 19-21, 84]. As a result, the numerous lineages allow for variability and adaptability which may contribute to the success of *Salmonella* I 4,[5],12:i:-. The distinct clones found in particular geographical regions with different R-types and deletions in the phase 2 flagellar region are indicative of the successful spread and dissemination of *Salmonella* I 4,[5],12:i:-. The evolution associated with the emergence and clonal expansion of the monophasic *Salmonella* Typhimurium strain can be evaluated using comparative whole-genome analyses as well as phylogenetic analyses. In our study, multiple-sequence alignments will be performed on *Salmonella* I 4,[5],12:i:- isolates to evaluate the deletion profiles of the phase 2 flagellar region and determine if there are any mobile genetic elements inserted in the region. Furthermore, a SNP-based phylogenetic analysis will be performed on *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates from our study as well as publicly available isolates from NCBI. This evolutionary analysis will allow us to evaluate the emergence of our *Salmonella* I 4,[5],12:i:- isolates from swine in the United States and will help determine the genetic relatedness with prevalent clones worldwide.

Table 2-1 Deletions in the phase 2 flagellar region, MLST, phage type, resistance type, and geographical regions of prevalent *Salmonella* I 4,[5],12:i:- clones

Clones	**MLST**	**Phage type**	**R-type**	***flj*B**	***flj*A**	***hin***	***iro*B**	**Geographical regions**
Spanish	ST19	U302	ACSSuT-GSxT	-	-	-	-	Europe
European	ST34	DT193/DT120	ASSuT	-	-	-	+	Europe/United States

United States	ST19/34	Susceptible	-	-	+	+	United States

Table 2-2 Resistance type, mobile genetic elements, and genotypic resistance of prevalent *S.* I 4,[5],12:i:- clones

Clones	R-type	MGE	Genotypic resistance
Spanish	ACSSuT-GSxT	Class 1 integrons/plasmids	*bla*$_{TEM-1}$, *cmlA1*, *aadA2*, *sul1*, *tet*(A), *aac(3)-IV*, and *dfrA12*
European	ASSuT	IS26 elements/plasmid replication genes/orfs (IncH1)	*bla*$_{TEM-1}$, *str*A-*str*B, *sul2* and *tet*(B)
United States	Susceptible	N/A	N/A

2.3. Swine as an important reservoir for Salmonella *4,[5],12:i:-*

2.3.1. Salmonella I *4,[5],12:i:- pathogenicity in swine*

Salmonella enterica is commonly found in swine and while infections are mostly subclinical, enterocolitis and septicemia may also occur [87]. The outcome of disease resulting from *Salmonella* in swine varies across serovars. *Salmonella* Choleraesuis is a host-adapted serovar that primarily causes septicemia in swine and is associated with high mortality and low morbidity rates [88]. Symptoms of a systemic infection includes fever, lethargy, anorexia, and dyspnea. Host-generalist *Salmonella* Typhimurium can cause significant disease in pigs such as enterocolitis, which is associated with low mortality and high morbidity infection rates. Enterocolitis symptoms include fever, lethargy, anorexia, and diarrhea. With enterocolitis, pigs can become chronic *Salmonella* carriers [89]. However, *Salmonella* Typhimurium has also been associated with subclinical infection, which displays no clinical signs or symptoms [90]. Therefore, pigs with subclinical

infection become asymptomatic carriers resulting in undetected transmission and contamination of pork products [91].

Salmonella Typhimurium is one of the most frequently isolated serovars in swine and pork products. Additionally, it has been isolated from the gastrointestinal tract and lymph nodes in both symptomatic and asymptomatic carriers [92, 93]. As discussed in the introduction, *Salmonella* I 4,[5],12:i:- is a monophasic variant of *Salmonella* Typhimurium that has been increasing in prevalence in swine. According to data from the National Veterinary Services Laboratory (NVSL), the isolation of *Salmonella* I 4,[5],12:i:- is becoming more common than *Salmonella* Typhimurium in the United States swine population [94]. Because flagella play an important role in the pathogenesis of *Salmonella,* the monophasic expression of *Salmonella* I 4,[5],12:i:- may impair its ability to infect swine and cause disease [95]. Therefore, identifying characteristics of *Salmonella* I 4,[5],12:i:- related to pathogenicity and survival are key to understanding its ecological success in swine. Flagella play in important role in motility and biofilm formation, which are key virulence factors involved in pathogenesis of *Salmonella*. Several *in vivo* studies have investigated the effect that monophasic expression of *Salmonella* I 4,[5],12:i:- has on its pathogenicity in swine [32, 33, 96, 97]. However, there are little to no studies investigating the *Salmonella* I 4,[5],12:i:- motility and biofilm production ability, *in vitro* or *in vivo*. Therefore, our investigations will determine the motility and biofilm production ability of monophasic *Salmonella* I 4,[5],12:i:- swine isolates *in vitro* and compare to

biphasic *Salmonella* Typhimurium swine isolates and other monophasic *Salmonella* serovars.

Two independent studies investigated and compared the ability of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from swine to infect porcine intestinal epithelial cells *in vivo*. Crayford et al. (2014) compared pig-derived DT193 *Salmonella* Typhimurium and DT193 *Salmonella* I 4,[5],12:i:- strains, while Shippy et al. (2018) compared a pig-derived virulent and susceptible *Salmonella* Typhimurium strain to a pig-derived MDR *Salmonella* I 4,[5],12:i:- strain from the multistate outbreak (Washington State, USA in 2015). Both studies found no significant differences in adhesion and invasion of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium [32, 96]. These findings indicate the monophasic expression of the phase 1 flagellar antigen (and lack of the expression of the phase 2 flagellar antigen) does not affect the pathogenicity of *Salmonella* I 4,[5],12:i:- during infection of porcine intestinal epithelial cells [32, 96]. Although these studies found *Salmonella* I 4,[5],12:i:- to have similar pathogenicity as *Salmonella* Typhimurium, other monophasic serovars were not included to determine if in fact the monophasic expression affects the pathogenicity of *Salmonella*. In our study, we will compare virulence factors of various monophasic serovars and *Salmonella* Typhimurium isolates collected from swine. One of the objectives of our study is to determine if the monophasic expression of *Salmonella* I 4,[5],12:i:- affects its motility and biofilm formation, and therefore it's virulence, which in turn may affect their ability to infect swine and cause disease.

Despite the fact that monophasic expression of *Salmonella* I 4,[5],12:i:- does not affect its pathogenicity in swine, studies have found differences in symptoms, fecal shedding, and histological lesions with *Salmonella* Typhimurium. In the study by Shippy et al. (2018), pigs inoculated with a pig-derived MDR *Salmonella* I 4,[5],12:i:- (the multistate outbreak strain) exhibited elevated rectal temperatures and diarrhea occurred one to two days post-infection (DPI) and after two DPI there were no clinical symptoms for the remainder of the trial. Continuous fecal shedding of *Salmonella* I 4,[5],12:i:- was observed with peak levels at one DPI and shedding decreased significantly by day seven. Shippy et al. (2018) also investigated tissue colonization of MDR *Salmonella* I 4,[5],12:i:- and observed higher levels of colonization in the intestinal tract (Peyer's patch region, ileocecal junction, and cecum) and the ileocecal lymph nodes than tonsils at 7 DPI [32]. Therefore, MDR *Salmonella* I 4,[5],12:i:- showed mild or self-limiting clinical disease in swine similar to that of a virulent non-MDR *Salmonella* Typhimurium strain from a previous study [32, 98].

Cevallos-Almedia et al. conducted two *in vivo* studies (2018 and 2019) with *Salmonella* I 4,[5],12:i:i- strains isolated from pig feces. They also observed elevated rectal temperatures and diarrhea occurring one to two days post-infection (DPI) in the first study [97]. However in the second study, Cevallos-Almedia et al. (2019) differed by observing no fever throughout the entire trial and diarrhea occurred late at 21 DPI [33]. Cevallos-Almedia et al. (2018) observed continuous fecal shedding with peak levels occurring three DPI which decreased significantly by day seven as seen in the Shippy et

al. (2018) study [97]. There are many other factors that contribute to *Salmonella* virulence and pathogenicity; thus, the virulence traits among *Salmonella* I 4,[5],12:i:- strains will vary. In contrast to *Salmonella* I 4,[5],12:i:-, pigs inoculated with the *Salmonella* Typhimurium showed intermittent shedding as seen in previous studies [33, 99, 100]. The two studies conducted by Cevallos-Almeida et al. also observed colonization of the gastrointestinal tract (duodenum, jejunum, ileum, and cecum) though tonsils were the most contaminated organ by 21 DPI [33, 97]. As mentioned, food animals, such as cattle and pigs, are often asymptomatic carriers of *Salmonella* [92, 93]. Food animals brought for slaughter can carry *Salmonella* in various tissues, such as the gastrointestinal tract and lymph nodes (e.g. tonsils), increasing the risk of carcass contamination at slaughter [92, 101]. In particular, lymph nodes including the tonsils, and peripheral and mandibular lymph nodes are inevitably included in cheek meat and head trim intended for ground meat leading to food safety concerns [102-105]. Importantly, our study will specifically investigate *Salmonella* isolated from market hogs' cheek meat and head trim intended for ground pork. Other variables such as the experimental pigs used, sample size, and *Salmonella* isolation in the study design may affect the results. Thus, the virulence and pathogenicity of *Salmonella* I 4,[5],12:i:- in swine is complex and many questions still remain.

A study by Arruda et al. (2019) solely focused on determining the pathogenic potential of *Salmonella* I 4,[5],12:i:- by evaluating and comparing microscopic intestinal lesions in swine enteric cases where *Salmonella* I 4,[5],12:i:- or *Salmonella* Typhimurium

was isolated [106]. Cases were randomly selected from the Iowa State University-Veterinary Diagnostic Laboratory database. A positive association was observed between intestinal lesions related to enteric salmonellosis and isolation of *Salmonella* I 4,[5],12:i:-. The majority of severe lesions caused by *Salmonella* I 4,[5],12:i:- were similar to swine enterocolitis cases from which *Salmonella* Typhimurium was isolated. However, *Salmonella* Typhimurium (94%) had a greater percentage of cases with lesions related to enteric salmonellosis than *Salmonella* I 4,[5],12:i:- (72%) [106]. Naberhaus et al. (2020) also evaluated gross lesions of pigs infected with *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium [34]. Importantly, *Salmonella* I 4,[5],12:i:- was isolated from lesions slightly less frequently than *Salmonella* Typhimurium, which is similar to the results by Aruda et al. and suggests *Salmonella* I 4,[5],12:i:- has a similar or possibly lower pathogenic potential than *Salmonella* Typhimurium in swine, which may provide an ecological advantage and facilitate its spread in swine and the environment.

Following host cell invasion, *Salmonella* triggers the host immune response via recognition of their pathogen-associated molecular patterns such as flagellin, (FliC and FljB), the major component of flagella [107]. Flagellin are recognized by receptors, such as Toll-like receptor 5 (TLR-5), which stimulate the production and release of proinflammatory cytokines and chemokines and activation of caspase [95, 108, 109]. Previous studies revealed *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium displayed similar levels of TLR-5 (flagellin receptor), IL-8 (proinflammatory cytokine), and INF-γ (proinflammatory cytokine) induction [32, 33, 96] in swine. Therefore,

expression of either flagellin is sufficient for porcine intestinal epithelial cell invasion and does not limit the ability to colonize tissue in pigs. The fact that *Salmonella* I 4,[5],12:i:- isolates have comparable virulence and pathogenicity as the more common serovar *Salmonella* Typhimurium, indicates *Salmonella* I 4,[5],12:i:- also has the potential to persist in swine throughout the pork production chain and eventually reach the slaughterhouse which increases the risk of *Salmonella* transmission and may lead to human infection through consumption of contaminated pork. In our study, we will evaluate *Salmonella* I 4,[5],12:i:- strains that have been isolated from head trim and cheek meat from asymptomatic market hogs at slaughter and are representative of strains that have successfully colonized swine which may enter the food chain.

2.3.2. Selection pressures: antibiotic and heavy metal use

Antibiotics and heavy metals are frequently used in food-producing animals. Antibiotics are used to prevent, treat, and control disease while heavy metals are used as antibiotic alternatives for growth promotion and to increase feed efficiency. In the 2019 Summary Report on Antimicrobials Sold or Distributed for Use in Food-producing Animals, the Federal Drug Administration (FDA) showed an increase in domestic sales and distribution of medically important antimicrobials that are approved for use in food animals [110]. In the swine industry, commonly used antibiotics such as ceftiofur, enrofloxacin, and tetracycline may be used to individually treat an animal or may be delivered by feed or drinking water to the entire farm/herd. However, the use of these antibiotics requires a prescription and supervision of a veterinarian. The Federal FDA

prohibited extra-label use of medically important antibiotics such as fluoroquinolones (e.g. enrofloxacin) and cephalosporins (e.g. ceftiofur) in 1997 and 2012, respectively. In 2017, the FDA implemented the Veterinary Feed Directive, which requires the supervision of veterinarians to use medically important antibiotics such as tetracyclines (e.g. chlortetracycline) in feed or water as they are no longer allowed for growth promotion. The use of ceftiofur and enrofloxacin in swine have important implications for human health as they belong to the same antibiotic class as ceftriaxone (third-generation cephalosporin) and ciprofloxacin (fluoroquinolone) which are used to treat severe *Salmonella* infections in humans. Cephalosporins and fluoroquinolones are classified as "critically important highest priority" antimicrobials by the World Health Organization [60]. Moreover, heavy metals such as zinc and copper are primarily used at high dietary levels in feed as an antibiotic alternative [111, 112]. The use of antibiotics and heavy metals in swine production allow for selection of AMR foodborne pathogens and transmission of AMR and heavy metal tolerance (HMT) genes. Consequently, resistance and tolerance to antimicrobials and heavy metals commonly used in swine provides a potential competitive advantage to *Salmonella* I 4,[5],12:i:- in the host and environment [77, 113].

A number of studies have identified various mutations and deletions (of the *flj*B gene, *flj*AB operon or surrounding genes) in *Salmonella* I 4,[5],12:i:- strains that have led to the lack of *flj*B (flagellar phase 2) expression. Mobile genetic elements that encode for antibiotic resistance and heavy metal resistance have been discovered within this region

[13, 114]. In *Salmonella* I 4,[5],12:i:-, common R-types found are ACSSuT-GSxT (Spanish clone) and ASSuT (European clone) [79, 82, 115]. The integration of AMR genes into the *flj*B region has been attributed to the integration of plasmids (e.g. IncH1), integrons (e.g. class 1), and insertion sequences (e.g. IS26) in prevalent clones. However, heavy metal resistance gene cassettes have also been identified within the *flj*B region alongside AMR genes. Therefore, the use of antimicrobials and heavy metals in the swine industry are potential selection pressures that may lead to incorporation of mobile genetic elements resulting in the emergence of MDR *Salmonella* I 4,[5],12:i:-.

2.3.2.1. Antimicrobial resistance and heavy metal tolerance in Salmonella I 4,[5],12:i:-

Elnekave et al. performed a comparative genomic (identification of AMR determinants and virulence genes) and phylogenetic analysis on *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates from different sources (livestock, human, and the environment) and locations (U.S. and Europe) in order to help explain the emergence of *Salmonella* I 4,[5],12:i:- [78]. The study focused on strains isolated from swine in the U.S. Midwest by the Minnesota Veterinary Diagnostic Laboratory (MVDL). The study population included MVDL clinical livestock isolates (from the years 2000 to 2015) and sequences obtained from the National Center for Biotechnology Information (NCBI). NCBI sequences included isolates from the United States and Europe, as well as multiple sources including livestock and humans (1991 to 2016). The first analysis of only MVDL *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates showed that the majority

(84%) of the *Salmonella* I 4,[5],12:i:- isolates collected from 2014 to 2015 were part of an emerging clade. Interestingly, within the emerging clade, *Salmonella* I 4,[5],12:i:- isolates were predominantly from swine (96%), ST34 (90%), R-type ASSuT (encoded by $bla_{\text{TEM-1}}$, *str*A-*str*B, *sul2* and *tet*(B)) (80%), and harbored the *Salmonella* genomic island 4 (SGI-4) (93%) [78]. SGI-4 was identified as a virulence factor encoding resistance to heavy metals such as copper and zinc in a previous study by Petrovska et al. [14]. On the other hand, the majority of *Salmonella* Typhimurium were ST19 and did not show the ASSuT resistance profile. Phenotypic resistance to enrofloxacin (22%) and ceftiofur (18%) was found in combination with the presence of plasmid-mediated resistance genes (*qnrB*19/*qnrB*2/*qnrS*1 and $bla_{\text{CMY-2}}$/$bla_{\text{SHV-12}}$, respectively). Higher similarity was also found between *Salmonella* I 4,[5],12:i:- from the emerging clade and *Salmonella* Typhimurium from Europe than with *Salmonella* Typhimurium from the United States [78]. Furthermore, chromosomal mutations in the genes encoding for the quinolone target enzymes, DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*) were discovered in 32 isolates. As mentioned earlier, *Salmonella* I 4,[5],12:i:- isolates with resistance or with reduced susceptibility to fluoroquinolones is slowly on the rise [35].

A comparative genomic study by Eleonora Mastrorilli et al. revealed the widespread presence of heavy metal tolerance gene cassettes in AMR *Salmonella* I 4,[5],12:i:- isolates [114]. A whole-genome analysis of 50 epidemiologically unrelated *Salmonella* I 4,[5],12:i:- strains from Italy (2010 to 2016) was performed to identify the presence of genetic elements conferring resistance to antibiotics and heavy metals. The

majority of strains (86%) contained heavy metal tolerance genes, *pcoA-pcoD* (copper) and *silA-silE* (silver), co-localized in the same genomic region; additional copper and silver genes were also found in this region (*cusR, cusB, cusA, cusF, cusC,* and *pcoE*). Interestingly, the tetracycline resistance gene *tet*(B) was co-localized in the same genomic region containing *pcoA, pcoD, silA,* and *silE* in 80% of the strains. About 54% of those strains also showed presence of *merA*, *merC, merP, merR,* and *merT* (mercury resistance genes); a few of which also contained tetracycline resistance genes *tet*(R), *tet*(C), and *tet*(A) within the same genomic region. In addition to heavy metal tolerance genes, 90% of *Salmonella* I 4,[5],12:i:- strains contained at least one AMR gene, though, the most common genetic AMR profile (84%) were MDR with the following genes, $bla_{\text{TEM-1}}$, *strA, strB, sul2, tet*(B), known to encode for the phenotypic ASSuT R-type. The majority of the strains carrying *sul2* were co-localized in the same genomic region which also contained the plasmid replicon IncQ1, indicating the resistance genes were transferred together via the plasmid. This study demonstrated a high prevalence of heavy metal tolerance genes (copper and silver) in *Salmonella* I 4,[5],12:i:- strains along with AMR genes. Several studies have found a strong association between antimicrobial resistance genes and heavy metal tolerance genes. The antimicrobial and/or heavy metal use provides a constant selection pressure for *Salmonella* resulting in the co-selection and acquisition of heavy metal tolerance and antimicrobial resistance when the genes are co-located on the same genetic elements such as plasmids, transposons or integrons [116, 117].

2.3.2.2. Antibiotic and heavy metal use in pigs

A common fluoroquinolone antibiotic used in agriculture animals, particularly in swine, is enrofloxacin. Enrofloxacin is approved by the FDA as an injectable solution (e.g. Baytril) for the control and treatment of bacterial swine respiratory disease (SRD) [23]. SRD is associated with *Actinobacillus pleuropneumoniae, Pasteurella multocida, Haemophilus parasuis, Streptococcus suis, Bordetella bronchiseptica* and *Mycoplasma hyopneumoniae*. In swine, enrofloxacin is also used for the control of colibacillosis associated with *E. coli* in groups or pens of weaned pigs. A single-dose is administered by intramuscular or subcutaneous injection and the withdrawal period is a minimum of five days before slaughter (FOI 141-068). Enrofloxacin administered to swine may contribute to the selection of fluoroquinolone resistant (or reduced susceptibility) *Salmonella* I 4,[5],12:i:- strains. Fluoroquinolone resistance genes often provide cross-resistance to both ciprofloxacin and enrofloxacin. Thus, it is a cause for concern as ciprofloxacin is a key fluoroquinolone antibiotic in treating invasive *Salmonella* infections in adults.

A study by Delsol et al. investigated the effect of a five-day enrofloxacin treatment on *Salmonella* Typhimurium DT104 strains in thirty-six experimentally infected pigs. There were three groups with 12 pigs each that were further divided into untreated (6) and enrofloxacin-treated (6) pigs. Each group was inoculated with three different ASSuT R-type *Salmonella* Typhimurium DT104 strains: 1) susceptible to nalidixic acid and ciprofloxacin, 2) cyclohexane-resistant strains (defined as low-level resistance to multiple antibiotics due to upregulation of efflux pumps), and 3) *gyrA* mutant strains resistant to nalidixic acid. There were no significant differences in fecal shedding between untreated

and treated pigs with quinolone-susceptible *Salmonella* Typhimurium DT104 strains possibly suggesting the enrofloxacin treatment or dose had no effect on susceptible strains. However, treated pigs inoculated with cyclohexane-resistant and nalidixic acid-resistant strains shed larger amounts of *Salmonella* than untreated pigs and the shedding remained elevated for two weeks or more [118]. The enrofloxacin treatment selected for quinolone-resistant *Salmonella* Typhimurium DT104 strains indicating there was a competitive advantage over susceptible *Salmonella* Typhimurium DT104 strains. As with most experimental studies, we do not know how other selection pressures or environmental factors play a role in the selection of resistant *Salmonella.* Currently, there are no reported experimental studies on the effect of enrofloxacin use in pigs on *Salmonella* I 4,[5],12:i:- and whether *Salmonella* I 4,[5],12:i:- quinolone-resistant strains have a competitive advantage over susceptible or quinolone-resistant *Salmonella* Typhimurium. In our study, we will investigate the differences in fitness *in vitro* between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from swine and with different genotypic quinolone resistant profiles in the presence and absence of antimicrobial selection pressures.

The 2015 NARMS Human Isolates Surveillance Report, noted an increase in the percentage of non-susceptible *Salmonella* strains to ciprofloxacin isolated from human clinical cases in the United States [28]. The use of enrofloxacin, a fluoroquinolone antibiotic, in animals such as swine may result in *Salmonella* strains that are fluoroquinolone-resistant or with decreased susceptibility to fluoroquinolones, which

potentially enter the food chain and lead to human infections. Fluoroquinolones act against *Salmonella* by inhibiting two enzymes, DNA gyrase and topoisomerase IV, essential for bacterial DNA replication. DNA gyrase is composed of two subunits (A and B) encoded by the *gyrA* and *gyrB* genes. Topoisomerase IV is also composed of two subunits (ParC and ParE) encoded by the *parC* and *parE* genes. Mechanisms of resistance to quinolones have been associated with chromosomal mutations in these genes resulting in target modification and in turn decreasing the affinity of their target sites (Table 2-2). Another mechanism of resistance is by acquiring plasmid-mediated quinolone resistance (PMQR) genes (*qnrA*, *qnrB*, *qnrC*, and *qnrS*) which encode proteins that bind and protect DNA gyrase and topoisomerase IV from fluoroquinolone activity (Table 2-2). Another PMQR gene is *aac(6')-Ib-cr*, which encodes a variant of an aminoglycoside acetyltransferase protein that acetylates the fluoroquinolones and decreases their activity. Increased active efflux by upregulation of porins is also a mechanism of resistance [119]. Individually, these mechanisms of resistance result to reduced susceptibility of fluoroquinolones. Clinical resistance to fluoroquinolones/quinolones requires the combination of point mutations in the target enzymes, active efflux, and/or acquisition of PMQR genes [120, 121].

In 2013, the Clinical and Laboratory Standards Institute (CLSI) revised ciprofloxacin minimum inhibitory concentration (MIC) breakpoints for *Salmonella*, including those that define resistant and intermediate categories of susceptibility. Decreased susceptibility to ciprofloxacin (MIC >= 0.12 μg/ml) is now used as a marker

for emerging fluoroquinolone resistance (CLSI, 2017). *Salmonella* infections with decreased susceptibility are important and have been associated with treatment failure and delayed antibiotic response [122, 123]. *Salmonella* I 4,[5],12:i:- with reduced susceptibility to ciprofloxacin have been detected in the U.S. Midwest, thus, are a public health concern [78].

Ceftiofur is a third-generation cephalosporin antibiotic also used in agriculture animals, such as cattle and swine. Ceftiofur use is approved by the FDA as an injectable solution (e.g. Excenel® and Excede®, Zoetis Animal Health, Florham Park, NJ) for the control and treatment of SRD associated with *Actinobacillus pleuropneumoniae, Pasteurella multocida, Streptococcus suis* and *Salmonella* Choleraesuis. In swine, Excenel (ceftiofur hydrochloride) is administered intramuscularly every 24 hours for a total of three consecutive days and the withdrawal period is no less than six days following the last treatment (FOI 141-288), while Excede (ceftiofur crystalline free acid) is administered as a single dose (FOI 141-235). In 2008, the FDA announced an order prohibiting the extra-label use of cephalosporins in food-producing animals. Ceftiofur administered to swine may contribute to the selection of ceftiofur resistant *Salmonella* I 4,[5],12:i:-. As with fluoroquinolones, this is a cause for concern as ceftriaxone (third-generation cephalosporin) is a key antibiotic in treating invasive *Salmonella* infections, primarily in children because of the toxic effects of fluoroquinolones. Thus, ceftiofur resistance genes often provide cross-resistance to ceftriaxone.

Several experimental and observational studies have been conducted on the selection pressure of ceftiofur on *E. coli* and *Salmonella* in both cattle and pigs [124-128]. An observational study by Lutz et al., investigated the association between different levels of ceftiofur use in 54 finishing swine barns and the recovery of ceftriaxone resistant *E. coli* or *Salmonella* strains [129]. Results showed barns with common and moderate ceftiofur use had greater odds of isolating ceftriaxone resistant *E. coli* than barns rarely using ceftiofur. In contrast, barns with rare and common ceftiofur use had similar odds of isolating ceftriaxone resistant *Salmonella* suggesting other factors may be involved in the selection or recovery of resistant *Salmonella* [129]. In a previous experimental model, pigs were inoculated orally with an extended-spectrum cephalosporin (ESC)-resistant *E. coli* strain containing a plasmid harboring the $bla_{\text{CTX-M}}$ gene. There were four distinct groups: control, ceftiofur-treated, *E. coli*-inoculated, and ceftiofur-treated/*E. coli* inoculated. In both of the non-treated and treated ceftiofur inoculated groups, *E. coli* persisted in most of pigs and there was indication that the $bla_{\text{CTX-M}}$ gene was transferred to other *E. coli*. Additionally, ESC-resistant *E. coli* strains were shed after the ceftiofur treatment and later there were no differences between the groups [127]. Similar findings were observed for *Salmonella* in an experimental study by Ohta et al. where beef cattle were administered ceftiofur [130]. Ohta's et al. study showed an increased proportion of MDR *Salmonella* in ceftiofur treated cattle indicating ceftiofur exerts selection pressure on *Salmonella* populations. However, similarly to *E.coli*, the prevalence of MDR *Salmonella* decreased by the second week [130]. Moreover, preexisting resistant bacteria within the population may be present and rapidly replicate in the presence of a particular antibiotic where the

majority of the susceptible population is killed; this phenomenon is known as heteroresistance [131].

Beta-lactams are one of the most important group of antibiotics that are widely used for bacterial infections due to their low toxicity, broad-spectrum and bactericidal activity. Therefore, resistance to beta-lactams is a public health concern. Beta-lactam antibiotics are characterized by having a common chemical feature known as the beta-lactam ring, that is highly reactive [132]. The class of beta-lactam antibiotics is further subdivided into subgroups: penems (e.g. aminopenicillins – ampicillin or amoxicillin), cephalosporins (e.g. 3rd generation ceftriaxone), carbapenems (e.g. meropenem), monobactams (e.g. aztreonam), and beta-lactamase inhibitors (e.g. clavulanic acid). Beta-lactams inhibit peptidoglycan synthesis by acting as structural analogs of penicillin binding proteins' (PBP) natural substrate, D-Ala-D-Ala dipeptide, and binding to the PBP active site [132-134]. Peptidoglycan is an essential component of the bacterial cell wall, and the inhibition of its synthesis leads to cell death. Mechanisms of resistance to beta-lactams includes: 1) inactivation of the antibiotic by beta-lactamases, 2) low-affinity PBP via modification, 3) increased efflux or decreased entry of antibiotic resulting in low concentration [133]. The most prominent resistance mechanism in *Enterobacteriaceae* is production of plasmid-encoded beta-lactamases such as TEM, SHV, CMY and CTX-M encoded by *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CMY}$, and *bla*$_{CTX-M}$ genes, respectively (Table 2-2). Variants of these beta-lactamases encode resistance to varying beta-lactams. For example, *bla*$_{TEM-1}$ and *bla*$_{SHV-1}$ encode for broad-spectrum beta-lactamases that confer resistance to

penicillins and older generation cephalosporins (e.g. first and second) while *bla*$_{\text{TEM-52}}$ and *bla*$_{\text{SHV-12}}$ encode for extended-spectrum beta-lactamases (ESBL) that confer resistance to penicillins, newer cephalosporin generations (e.g. third and fourth), and monobactams [135]. Pathogens encoding ESBLs, in particular, are of concern as they are resistant to a wide range of beta-lactams important in human medicine.

ESBL resistant *Salmonella* have been frequently recovered from humans, food animals, and retail meat with *Salmonella* Typhimurium and *Salmonella* Enteritidis being the most common serovars linked to ESBL resistance in human infections [136]. In the U.S., cephalosporin resistance in *Salmonella* has been primarily associated with poultry and predominantly due to beta-lactamases encoded by the *bla*$_{\text{CMY}}$ gene [137-139]. NARMS Now: Integrated Data includes 2007-2018 surveillance data from retail meats, animals, and humans. *Salmonella* I 4,[5],12:i:- isolates harboring the *bla*$_{\text{CMY-2}}$ gene have been mostly identified in retail poultry meat, but has also been found in market hogs and beef cattle cecal isolates as well as human stool isolates [140]. Although rare, *Salmonella* I 4,[5],12:i:- isolates have also been found to harbor the *bla*$_{\text{SHV-12}}$ and *bla*$_{\text{CTX-M}}$ genes. One human stool isolate has been found to harbor the *bla*$_{\text{CTX-M}}$ gene while only six *Salmonella* I 4,[5],12:i:- isolates with the *bla*$_{\text{SHV-12}}$ gene have been reported; three in market hogs, one in beef cattle, and two in human stool [140]. We will be determining the status of ESBL genes in *Salmonella* I 4,[5],12:i:- isolates are from market hogs. Furthermore, our study will investigate the fitness of *Salmonella* I 4,[5],12:i:- isolates in the absence or presence of ceftiofur and assess the potential fitness cost of ESBL resistance genes.

Tetracyclines (e.g. chlortetracycline and oxytetracycline) may be used as a feed additive to prevent gram-positive and gram-negative enteric infections in swine; however, a veterinarian's supervision is required due to the Veterinary Feed Directive (FDA 2015-13393). Oxytetracycline is used to treat bacterial enteritis caused by susceptible *E.coli* and *Salmonella* Choleraesuis as well as bacterial pneumonia caused by *Pasteruella multocida.* Oxytetracycline is fed continuously for one to two weeks and has a zero-day slaughter withdrawal period (FOI 008-804). Chlortetracycline is another antibiotic used in swine and may be paired with sulfathiazole and penicillin; it is used to prevent and treat bacterial swine enteritis caused by *Salmonella* Choleraesuis and vibrionic dysentery, reduce the incidence of cervical abscesses, and maintain weight gain of pigs with atrophic rhinitis. The recommended withdrawal period is seven to 15 days before slaughter.

Chlortetracycline used at subtherapeutic levels have been shown to increase the prevalence and proportion of resistant bacteria in pigs and cattle [130, 141]. Moreover, therapeutic levels of chlortetracycline in swine feed increased the proportion of tetracycline resistant *E.coli* [142]. Ohta et al. also observed an increase proportion of MDR *Salmonella* (including resistance to tetracycline) in chlortetracycline treated cattle. However, unlike the ceftiofur treated group in the Ohta et al. study, half of the *Salmonella* population in the chlortetracycline treated group remained MDR by day 26 [130].

In 2019, tetracyclines accounted for 67% of domestic sales and distributions of medically important antimicrobials approved for food animals, the most compared to other antibiotics [110]. The extensive use of tetracyclines are known to cause selection and

dissemination of resistant bacteria in the food industry. The mechanism of action of tetracycline antibiotics works by inhibiting bacterial synthesis. They attach to the ribosomal subunit and prevent the attachment of tRNA further inhibiting elongation during protein synthesis [143]. The most common mechanism of resistance to tetracycline in Gram-negative bacteria is through efflux pumps, which prevents accumulation of the antibiotic and in turn ribosomal binding. The most common tetracycline resistance genes encoding efflux pumps are *tet*(A), *tet*(B), *tet*(C), and *tet*(D) (Table 2-2). In *Salmonella*, the *tet*(A) gene has been detected on the chromosome and plasmids, while *tet*(B), *tet*(C), and *tet*(D) have been found on the chromosomes of several *Salmonella* serovars [144, 145]. Both *tet*(A) and *tet*(B) genes have been found in the Spanish and European *Salmonella* I 4,[5],12:i:- clone, respectively. The difference in tetracycline resistance genes and their fitness has not been explored in *Salmonella* I 4,[5],12:i:- strains. In our study, we will evaluate the fitness cost of tetracycline resistance genes found in *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains.

Overall, a strong correlation between antimicrobial use and the level of resistance in enteric bacteria has been well established [62, 146]. The administration of antimicrobials provides a selection pressure that favors expansion of antimicrobial resistant bacteria in the population. One of the objectives in our study is to investigate and compare the fitness of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates with different phenotypic resistance profiles under the selective pressures of ceftiofur, enrofloxacin, and tetracycline. Additionally, we will identify antimicrobial resistance

genes and determine if there is fitness cost of resistance by comparing the growth in the absence of antibiotics.

Heavy metals such as zinc and copper play critical roles in many biological processes and are needed for the survival of all living organisms [147]. As metal ions, zinc and copper act as structural components or regulatory co-factors for a variety of enzymes [148]. Food-animals such as pigs often receive zinc and copper in their diets through feed supplementation as micronutrients. The dietary zinc levels of 5 to 10 ppm and copper levels of 50 to 125 ppm are sufficient for the pigs' nutritional requirement for these processes. However, high concentrations (2000 to 3000 ppm for zinc and 100 to 250 ppm for copper) of the two metals are used for prevention of diarrheal disease, growth promotion and feed efficiency [77, 112]. The high levels of these micronutrients are eventually excreted through fecal matter and may accumulate and persist in the environment [149]. Although heavy metals are sometimes required by bacteria for essential cell functions, excess concentrations of zinc and copper can have significant toxicity to prokaryotic cells as they disrupt biological systems by inactivating cellular components such as nucleic acids, proteins, and lipids [148, 150]. As a result of the selection pressure, bacteria such as *Salmonella* develop ways to circumvent heavy metal toxicity by acquiring heavy metal tolerance genes. In our study, using WGS, we will determine the presence or absence of heavy metal tolerance genes in *Salmonella* I 4,[5],12:i:- isolates derived from swine.

Table 2-3 List of selected antimicrobial resistance genes encoding phenotypic resistance or reduced susceptibility to respective antimicrobial agents and the corresponding antimicrobial class

ANTIMICROBIAL CLASS		ANTIMICROBIAL AGENT	ANTIMICROBIAL RESISTANCE GENES
B-LACTAMS	**PENICILLINS**	Ampicillin (A)[1]	*bla*$_{CARB-2}$, *bla*$_{CMY-2}$, *bla*$_{TEM-1}$
	B-LACTAM COMBINATIONS	Amoxicillin/clavulanic acid (Au)[1]	*bla*$_{CARB-2}$, *bla*$_{CMY-2}$
	CEPHEMS	Cefoxitin (Cn)[1]	C2G: *bla*$_{CMY-2}$, bla$_{CTX-M}$
		Ceftriaxone (Cx)[1]	C3G: *bla*$_{CMY-2}$, bla$_{CTX-M}$, *bla*$_{SHV-12}$
		Ceftiofur (Cr)[2]	C3G: *bla*$_{CMY-2}$, bla$_{CTX-M}$, *bla*$_{SHV-12}$
AMINOGLYCOSIDES		Kanamycin (K)	*aac(3)-IId, aph(3')-Ia*
		Gentamicin (G)[1]	*aac(3)-IId, aac(3)-IV*
		Streptomycin (S)[3]	*aad*A, *strA* (aph(3')-Ib), *strB* (aph(6)-Id)
MACROLIDES		Azithromycin (Az)[1]	*mphA*
		Erythromycin (Er)	*ere*(A)
TETRACYCLINES		Tetracycline (T)[1]	*tet*(A), *tet*(B), *tet*(D), *tet*(G)
QUINOLONES AND FLUOROQUINOLONES		Ciprofloxacin (Cp)[1]	PMQR: *qnrB*2, *qnrB*19, *aac(6')Ib-cr* point mutations: *gyrA, gyrB, parC, parE*
		Enrofloxacin (E)[4]	
		Nalidixic acid (Na)[1]	
FOLATE PATHWAY ANTAGONISTS		Trimethoprim-Sulfamethoxazole (SxT)[1]	*dfrA12, dfrA19*
		Sulfisoxazole (Su)[1]	*sul1*, *sul2*, *sul*3
PHENICOLS		Chloramphenicol (C)[1]	*catA2, floR*

2.3.2.3. Co-selection of AMR and HMT genes

The use of heavy metals as antibiotic alternatives has raised concerns for co-selection of antimicrobial resistance in bacteria [116]. The genes encoding for

antimicrobial resistance and heavy metal tolerance are often co-located on the same mobile genetic element; therefore, selection for antimicrobial resistant strains co-selects for heavy metal tolerance and vice versa [151-153]. Previous studies have found a strong association between antimicrobial resistance and heavy metal tolerance in *Salmonella* isolates [154, 155]. Alternatively, other studies have shown that feeding high levels of dietary heavy metals is associated with an increase in AMR bacteria in swine [156, 157]. Furthermore, the genes encoding the ASSuT phenotypic resistance profile have been largely associated with class 1 integrons; thus, ampicillin, streptomycin, and sulfonamide resistance genes may be co-selected by the use of other antibiotics such as tetracycline [24]. Several studies have found a strong association between heavy metal tolerance and antimicrobial resistance in *Salmonella* isolated from swine in the USA including *Salmonella* Heidelberg, *Salmonella* Typhimurium, and *Salmonella* I 4,[5],12:i:- [77, 158, 159]. Mourão et al. was the first to report a high frequency of copper (*pcoA-pcoD*), silver tolerance (*silA-silE*), and mercury (*merA*) genes in MDR *Salmonella* I 4,[5],12:i:- clones [158]. The majority of Spanish *Salmonella* I 4,[5],12:i:- clones identified were found to harbor *silA-silE* (74%) and *merA* (91%) genes which were co-located on a non-transferable IncA/C plasmids and *sul3*-type III class integron with the ACSSuT-GSxT resistance genotype commonly known as the Spanish clone. While the majority of European *Salmonella* I 4,[5],12:i:- clones were also found to harbor *silA-silE* (98%), and *merA* (91%) genes, they also harbored *pcoA-pcoD* (98%). These heavy metal tolerance genes were co-located with the associated ASSuT resistance genotype on the chromosome [158]. Another study observed heavy metal tolerance genes *silA*, *pcoD*, and *merA* and

AMR genes *bla*$_{\text{TEM-1}}$, *str*A-*str*B*, sul2* and *tet*(B) were co-located on the chromosome of *Salmonella* Typhimurium (ST34) strains. Other *Salmonella* Typhimurium (DT104) showed presence of *bla*$_{\text{TEM-1}}$, *cmlA1, sul1, sul2, sul3* and *tet*(A) with *silA* on plasmid-borne *sul3*-type III class integron [113]. In addition to identifying presence of AMR and heavy metal tolerance genes in *Salmonella* I 4,[5],12:i:- isolates, we will investigate if these genes are co-localized on the same genetic element.

Salmonella I 4,[5],12:i:- infections have been largely associated with the consumption of pork and pork products; thus, swine have been identified as an important reservoir. In the swine industry, the use of antibiotics (e.g. enrofloxacin, ceftiofur, and tetracycline) and heavy metals (e.g. copper and zinc) exert selection pressures on bacterial communities like *Salmonella.* Therefore, one hypothesis is that these selection pressures may influence the transfer and integration of mobile genetic elements into biphasic *Salmonella* Typhimurium strains within the *flj*B region resulting in a monophasic variant with MDR and/or heavy metal tolerance. In addition, the successful spread has been associated with the increased incidence of antimicrobial resistance as well as heavy metal tolerance in *Salmonella* I 4,[5],12:i:- which facilitates the adaptation and expansion in swine and their environment [16, 17, 83, 160].

2.3.2.4. Fitness costs of antimicrobial resistance genes

Fitness is defined as the capability of an individual or genotype to replicate and survive in a competitive environment or population. Acquisition of AMR genes or chromosomal mutations are often associated with a fitness cost on the bacterium [161].

As a result, the fitness cost of resistance genes in the absence of the antimicrobial is often observed by reduced growth rate. Furthermore, the absence of antimicrobial(s) or reduction in antimicrobial use benefits more fit susceptible strains, which will outcompete less fit resistant strains over time. However, resistant bacteria can survive and replicate under antimicrobial selective pressures when resistant to respective antimicrobial [117]. Although most mechanisms of resistance have a fitness cost, there are exceptions where selection of low-cost resistance mutations may be beneficial or neutral [162, 163]. In addition, compensatory mutations, also known as secondary-site mutations, may re-establish or ameliorate fitness in the absence/presence of antimicrobials as well as increase the resistance level [162]. The stepwise selection of successive or compensatory mutations at non-lethal or sub-lethal selection pressures, such as antimicrobials used in food production animals, results in stepwise resistance [164]. The extent of the fitness cost is a significant biological parameter that impacts the rate of resistance gene transfer/mutations, rate of decreased resistance if antimicrobial use were reduced, and the stability of resistance [162]. Therefore, the relative fitness of AMR strains – in the absence and presence of an antimicrobial – is key in determining its evolutionary success. We aim to compare the relative fitness of *Salmonella* I 4,[5],12:i:- in the presence and absence of antimicrobials commonly administered in the swine industry with *Salmonella* Typhimurium and other monophasic *Salmonella* serovars.

There have been several studies measuring bacterial fitness by observing the growth and survival of resistant strains and wild-types under different conditions [165-

168]. In particular, the stepwise resistance of fluoroquinolones has been reviewed in *Salmonella* Typhimurium, *E. coli*, and *Streptococcus pneumoniae* [165-167]. A previous study investigated, *in vitro* and *in vivo* in a mouse infection model, the impact of bacterial fitness of fluoroquinolone resistant isogenic *E. coli* strains carrying up to five resistance mutations [165]. As expected, this study showed a decrease in fitness with individual mutations in the following genes: *gyrA1, gyrA2, parC, marR* and *acrR.* The *marR* gene encodes for multiple antibiotic resistance protein MarR which is the repressor of the marRAB operon involved in the activation of AMR and oxidative stress genes [169]. The *acrR* gene encodes for a regulator protein for the AcrAB subunits of the AcrAB-TolC multidrug efflux pump [170]. As the number of resistance mutations increased, particularly after the third mutation, increased resistance and fitness was observed both *in vitro* and *in vivo*. Similar results were observed in *Streptococcus pneumoniae* for *in vitro* fitness experiments with double mutants of *gyrA, gyrB, parC,* and/or *parE* [166]. These results indicate, at least for fluoroquinolones, the selection for improved fitness may drive the selection for resistance mutations, which in turn increase resistance to fluoroquinolones. Our study will be the first known study to compare fitness costs of AMR genes found in pig-derived *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains *in vitro*.

Antimicrobial resistance is often associated with a decrease in fitness [162, 164]. This is important since *Salmonella* I 4,[5],12:i:- are mostly MDR and have been found to have reduced susceptibility to fluoroquinolones. Additionally, as described earlier,

Salmonella I 4,[5],12:i:- have similar virulence in swine as *Salmonella* Typhimurium, which possibly means they have similar fitness levels to their biphasic relative. However, the fitness of a drug resistant strain largely depends on the resistance mutation and mechanism, stability of the resistance, and the antimicrobial concentration [164]. Thus, one of the objectives of our research is to determine the relative fitness and costs of associated resistance genes of *Salmonella* I 4,[5],12:i:- strains isolated from swine under antibiotics commonly used in swine production at varying concentrations (e.g. ceftiofur, enrofloxacin, and tetracycline). Because most successful clones are those where high-level resistance can be obtained with little to no loss in fitness, it is important to determine if MDR *Salmonella* I 4,[5],12:i:- have associated fitness costs [165, 166, 171]. Although resistance is known to aid in the selection, survival, and propagation of bacterial strains, particularly those under selection pressures, the link between resistance, fitness cost, and selective advantages remains unclear for *Salmonella* I 4,[5],12:i:-. Therefore, our study will help identify the fitness costs and/or benefits of a particular antimicrobial resistance genes that allow us to further understand the evolutionary success of *Salmonella* I 4,[5],12:i:- in swine and their environment.

2.3.3. Salmonella *I 4,[5],12:i:- in pork production chain*

As discussed earlier, pork and pork products have been implicated as an important source in foodborne outbreaks related to *Salmonella* I 4,[5],12:i:- [23, 24, 65, 67]. Previous studies have reported *Salmonella* I 4,[5],12:i:- at different stages in the pork production chain (e.g. farrowing, weaned, and finishing pigs and carcasses) and the surrounding

environment (e.g. feed, farms, and abattoir) along with other common serovars found in swine (e.g. *Salmonella* Typhimurium, *Salmonella* Derby, *Salmonella* Rissen) [172-175]. Our *Salmonella* I 4,[5],12:i:- strains, in particular, originate from cheek meat and head trim tissue of swine head carcasses from a large pork processing plant in the southern U.S.

Casanova-Higes et al. evaluated the prevalence of *Salmonella* isolated from weaned piglets and sows from five *Salmonella*-seropositive breeding farms in Spain for a period of one year [172]. Gastrointestinal samples, including mesenteric lymph nodes (MLN) and intestinal content (e.g. feces from cecum to rectum), were collected from male, weaned piglets intended for slaughter and female, weaned piglets raised as gilts to detect *Salmonella*. *Salmonella* I 4,[5],12:i:- was the most common serovar in piglets from both sample types and was found in all farms, which is expected as *Salmonella* I 4,[5],12:i:- is the top serovar isolated from swine in Spain. Other serovars detected were *Salmonella* Rissen, *Salmonella* Bovismorbificans, and *Salmonella* Derby [172].

Another one-year longitudinal observational study of five farrow-to-finishing farms in Australia investigated the occurrence and persistence of *Salmonella* I 4,[5],12:i:- , where it had been previously isolated [176]. Pooled fecal samples were taken from gestating sows, lactating sows and litters, weaners, and finisher pigs. *Salmonella* I 4,[5],12:i:- was detected across all farms with a higher proportion in weaner and finisher pigs shed samples than sow and farrowing pig samples. Both of these studies suggest *Salmonella* I 4,[5],12:i:- has the ability to colonize and persist in market hogs, which become asymptomatic carriers, at different stages throughout the production chain.

Moreover, the presence of *Salmonella* I 4,[5],12:i:- at later stages in the market hogs' life cycle is a potential food safety risk as the pigs are practically market ready.

Several studies in the U.S. have established the presence of *Salmonella* in each stage of the pigs growing cycle, yet none of the studies have isolated or reported *Salmonella* I 4,[5],12:i:- specifically at swine production sites [177-181]. For example, in 2006, the USDA's National Animal Health Monitoring System (USDA-NAHMS) Swine study randomly sampled feces collected from pens with grower and finisher pigs (containing 100 or more pigs) to detect the presence of *Salmonella* at pork production sites across the U.S. Midwest, Northcentral, and Southeast regions (included 135 sites in 17 states) [178]. The selected states were representative of approximately 94% of the U.S. pork producers and U.S. pig inventory. The top three serovars detected were *Salmonella* Derby, *Salmonella* Typhimurium, and *Salmonella* Agona which remained the same compared to the past two previous studies (1995 and 2000) [178]. Additionally, a longitudinal study sampled piglets, sows, nursery, and finisher pigs from 30 commercial swine farms in the North Carolina, (Southeast); which found *Salmonella* Typhimurium to be the most predominant serovar followed by *Salmonella* Infantis and *Salmonella* Anatum at the farm-level [181]. *Salmonella* I 4,[5],12:i:- has been reported at Veterinary Diagnostic Laboratories (VDL) isolated from swine clinical samples (2008 to 2017) originating from different swine production systems, including at Iowa State VDL and Minnesota VDL (both Midwest regions) [182, 183]. Naberhaus's et al. discovered *Salmonella* I 4,[5],12:i:- had been the most common serovar found in swine clinical

samples at the Iowa State VDL, while *Salmonella* Typhimurium was the second most frequent serovar from 2008 to 2017 [182]. Furthermore, the Minnesota VDL noted an increase in *Salmonella* I 4,[5],12:i:- as well as *Salmonella* Agona in swine clinical samples from 2006 to 2015, while there was a decrease *Salmonella* Typhimurium and *Salmonella* Derby [183]. There are many factors why the detection of *Salmonella* I 4,[5],12:i:- may vary at different stages of the pork production chain including but not limiting to geographical location, type of farms (e.g. wean-to-finish or finishing farms), seasonality ,as well as sampling methods, duration of study, and culturing isolation method of *Salmonella*. The *Salmonella* I 4,[5],12:i:- isolated in our study were obtained from swine head carcasses tissue samples from a southern pork processing plant that services several market hog farms across the Midwest U.S.

As mentioned, swine are an important reservoir for *Salmonella,* particularly *Salmonella* I 4,[5],12:i:-, and often act as asymptomatic carriers. Subclinically infected swine taken to slaughter may carry *Salmonella* in various tissues such as the gastrointestinal tract and lymph nodes (e.g. tonsils), increasing the risk of carcass contamination at slaughter [92, 101, 184]. A previous study characterized *Salmonella* contamination on swine carcasses from two large pork processing plants in the U.S. by sampling the surface of carcasses before scalding (pre-scald), after dehairing/polishing (pre-evisceration), and after chilling [185]. The overall *Salmonella* prevalence was greatest at pre-scald (~91%) compared to pre-evisceration (19.1%) and after chilling (3.7%). As expected, the scalding and dehairing/polishing (also includes antimicrobial

surface treatments) washes and cleans the hide or skin on the carcass before evisceration, decreasing the prevalence of *Salmonella* on swine carcasses. Furthermore, *Salmonella* Derby, *Salmonella* Typhimurium, and *Salmonella* Anatum serovars were the most common detected serovars pre-scalded (~40 serovars detected). *Salmonella* Typhimurium remained predominant at pre-evisceration (24 serovars) and after chilling (9 serovars) [185]. Also, over 76% of *Salmonella* Typhimurium strains were MDR showing the ACSSuT phenotype. The vast majority of market hogs entering the two U.S. pork processing plants showed high *Salmonella* prevalence, which is not uncommon as transportation from farm to the abattoir, environmental contamination, and housing in the same pen with other possibly infected pigs from other farms may be possible sources of cross infections of *Salmonella* [186]. Additionally, pigs are known to become stressed during loading, transport to abattoir, and unloading of pigs due to unfamiliar noise, smells, temperature, and compact grouping; as a result, studies have shown an increase amount of *Salmonella* fecal shedding, as well as, higher rates of isolation after transport and at slaughter when compared to rates on the farm [89, 179]. It is also important to note that although the prevalence of *Salmonella* was low after chilling, the presence of MDR *Salmonella* Typhimurium along with a few other serovars (e.g. Johannesburg and Derby) suggests persistence of these serovars throughout the slaughter process to retail meat. In our study, the presence of *Salmonella* I 4,[5],12:i:- in swine head carcasses (post-evisceration) suggests successful colonization and persistence in swine similarly to *Salmonella* Typhimurium.

In general, it is known that lymphatic tissues are present in cuts of retail meat intended for ground product. *Salmonella* in lymph nodes are naturally encased in fat which protects against antimicrobial surface treatments and other interventions of food animal carcasses [93]. Moreover, the increased risk of *Salmonella* contamination may be due to the higher prevalence in lymph nodes over other tissues (e.g. spleen) [187]. *Salmonella* has been found in swine lymph nodes associated with retail meat cuts of carcasses at abattoirs [93, 104, 174, 180]. Bessire et al. investigated the *Salmonella* prevalence in lymph nodes (superficial inguinal lymph nodes) of market hogs and sows (21 pork harvest facilities in the northern and southern U.S.) and found the overall hog-type prevalence of *Salmonella* was 20.5% for sows and 8.6% for market hogs [93]. Another study in Mexico, also evaluated the prevalence of *Salmonella* in lymph nodes (e.g. mandibular and mesenteric lymph nodes) and tonsils of swine carcasses from two mixed beef/swine abattoirs. *Salmonella* was present in all tissues sampled with the highest prevalence in tonsils (40%) at one abattoir and mesenteric lymph nodes (44.4%) at the other. In addition, prevalence in the mandibular lymph nodes ranged from 12.7% to 20%. Furthermore, multiple lymph nodes (e.g. mandibular and peripheral lymph nodes), as well as the tonsils, are often found in head trim and cheek meat intended for ground pork [102-105]. After the slaughtering process of market hogs, the swine head is removed for post-mortem inspection for any indication of disease such as inflammation, swelling, cysts, lesions, and pathology of lymph nodes such as the mandibular or peripheral lymph node (FSIS-USDA Post-mortem livestock inspection directive). A study conducted by Harvey et al. (2017), sampled a large pork processing plant in the U.S. and found the overall prevalence of

Salmonella from swine carcass heads was 64.5%, while the tissue-specific prevalence for head trim was 66.22% and cheek meat was 62.83% [174]. It was also observed that out of 774 *Salmonella* isolates, about 50% of the isolates were *Salmonella* Typhimurium and *Salmonella* Typhimurium had the highest prevalence at 24.92%. Among the monophasic strains, *Salmonella* I 4,[5],12:i:- had the highest prevalence (3.92%) (although it is relatively low in comparison to Typhimurium), followed by Derby (1.66%) and Senftenberg (1.33%) [174]. These studies indicate that swine can carry *Salmonella* in lymph nodes that may incorporated in pork and pork products; thus, serving as a potential source of *Salmonella* contamination in food products and leading to foodborne illness in humans. Our *Salmonella* isolates are derived from the Harvey et al.'s study are and were used to further investigate the clonality and success of *Salmonella* I 4,[5],12:i:- in swine.

Overall, these studies suggest that the farm and slaughter environment play a key role in the persistence and dissemination of *Salmonella* in swine throughout the pork production process. Therefore, the pork production food chain is an important reservoir of successful *Salmonella* serovars, including *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:-. In particular, the finishing phase and slaughter seem to be significant sources of *Salmonella* I 4,[5],12:i:- as this serovar was found in market ready hogs and swine carcasses, respectively. This is a public health concern as previously mentioned because *Salmonella* I 4,[5],12:i:-, often MDR, has been implicated in foodborne outbreaks associated with the consumption of contaminated pork. Thus, my study will improve upon the understanding of the ecological success of *Salmonella* I 4,[5],12:i:- in swine and its

ability to cause infection in humans by exploring the differences in pathogenicity and virulence traits between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolated from swine at slaughter.

2.4. Important Salmonella *virulence factors*

2.4.1. Salmonella *flagella*

2.4.1.1. Motility

Many microorganisms that cause disease, including *Salmonella enterica* and *Escherichia coli*, are motile. The motility of a bacterium is related to the use of a specialized rotating organelle known as the flagellum [37]. Flagella facilitate the motility of an organism within its environment which allows it to move towards attractants (e.g. searching for nutrient sources) and away from repellants (e.g. avoiding toxic compounds) via chemotaxis. Additionally, flagella are known to be involved during the initial stages of infection. Therefore, flagella have a major role in the pathogenicity of *Salmonella* and are considered to be an important virulence factor.

There are two distinct mechanisms of *Salmonella* motility: swimming and swarming. Swimming is an individual cell movement in liquid environments powered by short vegetative rotating flagella, and swarming is a multicellular bacterial surface movement powered by elongated and hyperflagellated rotating flagella [41, 188]. In order for swarming to occur, *Salmonella* swimmer cells undergo morphological differentiation into multinucleated and hyperflagellated swarmer cells. In both cases, motility is enabled by flagella emerging from the cell surface driven by a motor at their base [189]. Swimming

motility is closely related to chemotaxis, which is the ability to orientate along certain chemical gradients. The motor and the base of the flagella are linked to a molecular single transduction cascade that senses chemical gradients and transmits signals of attraction or repulsion to the flagellar motor, which will then react by altering its rotation direction [37]. The flagella allow *Salmonella* to avoid or acclimate to unfavorable environments and to reach, as well as maintain ideal environmental niches for colonization.

Brunelle et al. evaluated the effect of antibiotics on the swimming and swarming motility of MDR *Salmonella* isolates. Antibiotics such as chloramphenicol and tetracycline decreased swim and swarm motility. In comparison, kanamycin and streptomycin slightly decreased swimming and had little to no effect on swarming [190]. Interestingly, *Salmonella* Typhimurium differentiation into swarmer cells have shown to elevated resistance to kanamycin as well as nalidixic acid, ciprofloxacin, streptomycin, and colistin [188]. These studies demonstrate how antibiotics impact motility in *Salmonella* Typhimurium strains. However, there are no studies comparing the swimming and swarming motility of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains nor the differences among MDR strains. Therefore, our study will investigate the differences in swimming and swarming motility between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates, as well as other monophasic strains. Flagella-mediated motility enables *Salmonella* to search for nutrients, avoid toxic compounds, initiate biofilm formation, and most importantly colonize new niches [37]. Thus, assessing the motility of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium successfully isolated

from market hogs at slaughter will help us understand the role flagella and phase-variation in host colonization.

2.4.1.2. Flagellar phase variation

The flagellum is a long helical, rotatable appendage, approximately 15 μm in length, located across the cell surface [191]. The flagellum is composed of three structural components: the basal body, a hook, and the filament. The filament is a rigid structure with a long helical shape that functions as a propeller. The filament is composed of thousands of subunits of FliC or FljB flagellin proteins (flagellar antigens) and capped at the tip by the FliD protein to prevent flagellin subunits from leakage of unassembled flagellin monomers [192]. Flagellum biosynthesis is a highly ordered process that requires expression of numerous genes for assembly and function of flagella, including structural components of flagella, regulators, and chaperones.

Phase variation is a common phenomenon of bacteria such as *S. enterica* that helps the bacteria to survive in harsh or changing environments. Phase variation results in phenotypic heterogeneity (58–60). In terms of cell envelope components, phenotypic heterogeneity makes it possible for bacteria to evade or modulate the host immune system without restriction of the pathogens or requiring the pathogen to acquire new genes (58, 60–63). *Salmonella* Typhimurium has been known to switch between two antigenic forms of flagellin filament protein, type B (FljB) or C (FliC) flagellin. Stochastic inversion of a promoter producing both FljB and an inhibitor (FljA) of type C flagellin formation leads to the phenomenon of flagellar phase variation (Figure 2-3) [12].

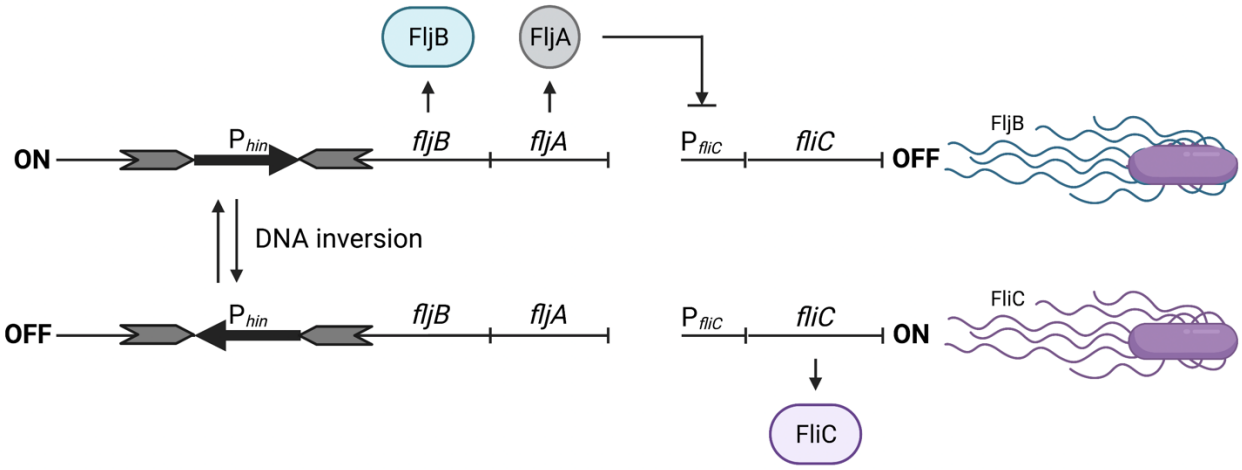


Figure 2-2 Schematic representation of flagellar phase variation in *S. enterica* adaptation from Bonifield and Aldridge et al. created with Biorender.com [12, 193]

2.4.2. Biofilms

2.4.2.1. Role in food safety and human disease

Biofilm formation is an important virulence factor in several bacterial species, including *Salmonella*, as it provides resistance to chemical, physical, and mechanical stresses in the environment or within a host such as the hosts' immune response. Biofilms also provide resistance to antimicrobial agents used to treat infection, as it serves as a barrier which prevents or reduces the chance of contact with the antimicrobial agent; thus, influencing the outcome of an infection [193, 194]. Biofilms play an important role in infections and in the U.S. and approximately 80% of persistent infections are associated with biofilms [195, 196].

Furthermore, biofilms are a concern for food safety and hygiene, as the ability of bacteria to form biofilms allows for attachment and persistent growth on biotic and abiotic

surfaces under suitable conditions such as food products and food contact surfaces [197]. Food contact surfaces include plastics, metal, glass or rubber which are normally encountered in farms, the food processing industry (e.g. slaughterhouses), and kitchens [43]. This allows for foodborne pathogens like *Salmonella enterica* or *Staphylococcus aureus* to persist from farm to fork outside a host. Biofilms lead to hygiene issues, food spoilage, and consequently, human foodborne illnesses [198]. During meat processing such as at a slaughterhouse, biofilm accumulation may occur on the floors, stainless steel surfaces of tools or equipment and provide a potential source of cross contamination [198]. Biofilms are also responsible for a various infections in veterinary medicine [199]. Biofilms are important in establishing chronic infections in humans and animals, as well as, for persistence in the environment.

2.4.2.2. Biofilm formation

Biofilm formation is influenced by the structural composition, bacterial genome, environmental stimuli, and stressors [200, 201]. Biofilms are formed in a multistep and highly regulated process that includes reversible attachment to a surface, irreversible attachment by binding with adhesions or exopolysaccharides, development of microcolonies, biofilm maturation, and dispersion [200]. In the first stage of reversible attachment, planktonic cells weakly adhere to a surface mediated by surface appendages such as flagella, pili, curli, and outer membrane proteins. In the second stage of irreversible attachment, the formation of a monolayer occurs by permanently bonding to a surface with exopolysaccarides [198]. During microcolony formation, swimming motility is inhibited,

and the accumulation and growth of microorganisms occurs by replication and recruitment of planktonic cells via quorum sensing (cell-to-cell communication). The stage of biofilm maturation is associated with the production of the extracellular matrix, composed of exopolysaccarides, O-antigen capsule, and biofilm-associated proteins (BAPs), which encase the microorganisms. The extracellular matrix provides both structure and protection in harsh environments. The final stage in biofilm formation is dispersion. In this stage, the matrix bound microorganisms may detach and revert into their planktonic form and disperse into the environment [198]. Since flagella are known to facilitate biofilm formation during the initial stage by promoting surface binding which in turn aids in adhesion during host colonization, it is important to understand how the expression of flagella in monophasic and biphasic *Salmonella* may affect biofilm formation.

In *Salmonella*, biofilms are primarily composed of curli fimbriae, cellulose, BAPs, the O-antigen capsule and extracellular DNA [43]. The expression of these important components contributing to biofilm formation are often serovar specific in *Salmonella,* dependent on the properties of the surfaces, and dependent on nutrient availability. Several studies have shown that *Salmonella* are capable of forming biofilms on plastic surfaces [202-204]. A study by Yin et al. investigated the ability of several *Salmonella* serovars (*S.* Typhimurium, *S.* Senftenberg, *S.* Derby, *S.* Agona, and *S.* Kingston) isolated from beef processing plants, to form biofilms under various conditions (e.g. temperatures and pH). Biofilm formation was assessed using the crystal violet assay and the relationship between biofilm formation and the presence of biofilm-related genes was evaluated (e.g. *adrA*,

fimH, *csg*A, *csgB*, *csgD*, *csrA*, *sirA*, *glyA*, *ompR*, *sdiA*, *sipB*, *sipC*, *luxS*, *pfs*, and *gcpA*). All *Salmonella* strains possessed the ability to form biofilms on polystyrene surfaces at 77 to 98.6°C. However, the ability to form biofilms differed among serovars with *S.* Senftenberg and *S.* Kingston having a greater biofilm formation ability than *S.* Agona [204]. These findings are consistent with previous studies which have found variability in biofilm formation among *Salmonella* serovars. For example, a study by Agarwal et al. found the majority (99.34%) of *Salmonella* strains (encompassing 69 serovars), showed the ability to form biofilms on a plastic surface with the majority being moderate (57.61%) biofilm producers, followed by weak (22.52%) and strong (19.21%) biofilm producers [205].

Although there are a number of biofilm formation studies related to *Salmonella* Typhimurium, there are currently only two studies focusing on *Salmonella* I 4,[5],12:i:-. One study demonstrates the ability of *Salmonella* I 4,[5],12:i:- strains (from clinical, environmental, and veterinary samples in Portugal) to form biofilms on plastic surfaces after 24 hours [206]. Approximately half of the isolates were weak biofilm producers while the other half were moderate producers at 24 hours, while moderate biofilm production was observed at 48 (66.2%) and 72 (69.2%) hours. However, this study does not compare *Salmonella* I 4,[5],12:i:- to *Salmonella* Typhimurium strains. The second study did compare biofilm formation among *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from different farms, production stages, and sources in Ireland [207]. No significant differences were observed in biofilm formation between the

two serovars at 24 and 48 hours. Though there was no differences, *Salmonella* Typhimurium strains (strong, moderate, and weak biofilm producers) showed a greater variation in biofilm formation than *Salmonella* I 4,[5],12:i:- (moderate producers). Also, biofilm-related genes (100% *adrA,* 100% *csgD,* and 97% *gcpA*) present in nearly all isolates did not have a significant association to a biofilm-positive phenotype. The results of these studies suggests that biofilm formation is not affected by the inability of monophasic *Salmonella* I 4,[5],12:i:- strains to express the phase 2 flagellar antigen. Only one of these studies looked at biofilm-related genes (*adrA, csgD,* and *gcpA*). Our study will improve upon this information by evaluating the relationship between biofilm production and the presence of several other biofilm-related genes, including *adrA*, *fimH*, *csg*A, *csgB*, *csgD*, *csrA*, *sirA*, *glyA*, *ompR*, *sdiA*, *sipB*, *sipC*, *luxS*, *pfs*, and *gcpA*.

Since the ability to form biofilms is thought to be key in persistent infections and survival, it is important to determine the biofilm-forming ability of *Salmonella* I 4,[5],12:i:- in comparison to biphasic *Salmonella* Typhimurium isolated from swine in the United States to understand if this is another potential virulence trait that provides a competitive advantage in swine and their environment. Our study will investigate the ability of monophasic and biphasic *Salmonella* strains to form biofilms and determine the association of biofilm production with the presence of biofilm-associated genes.

2.5. Typing of **Salmonella** *I 4,[5],12:i:-*

2.5.1. Traditional serotyping

Traditional serotyping of *Salmonella* has been used for several years as a means for monitoring infections and understanding the epidemiology of important *Salmonella* serovars involved in outbreaks. *Salmonella* are traditionally classified into serovars based on a phenotypic subtyping method well-established by the World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella*, The Pasteur Institute [9]. The serological method, called the White-Kauffmann-Le Minor serotyping scheme, uses agglutination tests with specific antisera to determine expression of the O (somatic) and H (flagellar) antigens [9].

The O-antigen is a component of lipopolysaccharides (LPS) located on the outer most surface of the bacterial cell wall, consisting of oligosaccharides repeats of two to eight sugars. The O-antigen is highly variable as it differs in structure and composition across bacterial species and strains [208, 209]. The variability is a result of the genetic diversity within the *rfb* (O-antigen) gene clusters, which are responsible for O-antigen synthesis [210]. The flagellar antigenic differences are due to the highly variable central region sequences of flagellar antigen genes. A serovar is represented by their antigenic formula indicating the O, phase 1 flagellar, and phase 2 flagellar antigens and are separated by a colon in their respective order.

Aforementioned, *Salmonella* Typhimurium can alternately express both the phase 1 flagellar antigen (i) and phase 2 flagellar antigen (1, 2). Thus, the antigenic formula for *Salmonella* Typhimurium is 4,[5],12:i:1,2. For monophasic *Salmonella* Typhimurium, only expressing phase 1, the antigenic formula is 4,[5],12:i:-. Both contain the same O

antigens, 4,[5],12. Identification of *Salmonella* I 4,[5],12:i:- has been difficult. After the somatic antigens of serogroup B and the phase 1 flagellar antigen have been identified, phase inversion assays must be performed repeatedly for the confirmation of the presence or absence of the phase 2 flagellar antigen. When the phase inversion is negative, there is a possibility of weak expression of the phase 2 flagellar antigen. The assays are time consuming and take several days for full determination and there is no set number of how often the assays should be repeated to ensure a strain is monophasic [211]. There is a possibility of misclassification of monophasic variants of *Salmonella* Typhimurium. In addition to misclassification, disadvantages of this method include quality control of hundreds of antisera, cost of antisera, and required experience. Traditional serotyping is usually limited to reference laboratories [211-213].

The misclassification of *Salmonella* I 4,[5],12:i:- has led to other methods such as WGS or polymerase chain reaction (PCR) to confirm the lack of the phase 2 flagellar antigen gene or other genes involved in flagellar antigen expression. Because most *Salmonella* I 4,[5],12:i:- are multidrug-resistant (including antibiotics used to treat *Salmonella* infections in humans) when compared to *Salmonella* Typhimurium, the characterization of monophasic *Salmonella* I 4,[5],12:i:- strains is essential to provide the appropriate clinical and regulatory action. There are many *Salmonella* serovars; however, a small number of serovars of *Salmonella* are responsible for the majority of cases of human salmonellosis [58]. Different serovars have different host-specificity and virulence traits including the ability to infect different hosts and cause disease. Therefore, proper

identification of *Salmonella* serovars is important for surveillance, outbreak investigations, and mitigation strategies.

2.5.2. Whole-genome sequencing-based serotyping

WGS is quickly replacing the current serotyping and subtyping methods for surveillance and outbreak investigations of foodborne pathogens, including *Salmonella* I 4,[5]12:i:-. Not only does WGS allow for high-resolution typing, WGS provides valuable data of *Salmonella* strains such as presence or absence of antibiotic resistance determinants, heavy metal tolerance genes, plasmids, and virulence genes; it also allows for confirmation of traditional serotyping results. WGS-based serotyping methods, also referred to as *in silico*-based serotyping, can be performed using free available software such as SeqSero with raw or assembled sequencing reads [214]. Although assembly of raw sequences are not required, high-throughput genome sequencing data are needed for accurate serovar identification. This method maps sequencing reads to curated databases of genetic serovar determinants. The determinants include the *rfb* gene clusters encoding O-antigens, *wzx* and *wzy* genes encoding flippase and polymerase, and *fliC* and *fljB* encoding flagellar antigens [215].

While there is a high agreement of phenotypic and genotypic serotyping methods, misclassifications of atypical monophasic *Salmonella* I 4,[5]12:i:- strains may occur as they are determined by the lack of *fliC* or *flj*B genes and not by point mutations or disruptions of other important genes involved in flagellar expression and/or flagellar phase variation [19, 216, 217]. Because serotyping monophasic variants of *Salmonella*

Typhimurium can be challenging, often both phenotypic and genotypic serotyping are performed for accurate identification, as in our study.

2.5.3. Genotyping via polymerase chain reaction

Similar to WGS, PCR can be combined with traditional serotyping to identify and differentiate *Salmonella* Typhimurium and *Salmonella* I 4,[5]12:i:- strains [218]. The PCR assay aims to simultaneously detect the fliB-fliA intergenic region and the *fljB* gene. *Salmonella* Typhimurium and its monophasic variant possess a 1 kb fliB-fliA intergenic region with the IS200 fragment. The IS200 fragment is unique to *Salmonella* Typhimurium and its monophasic variants. Thus, the IS200 fragment is not detected in the other serovars, and fliB-fliA intergenic region is only 250 bp. In addition to the fliB-fliA intergenic region, *Salmonella* Typhimurium and other serovars show a 1389 bp fragment for the *fljB* gene. *Salmonella* I 4,[5]12:i:- are classified as monophasic when the *fljB* is not detected [219, 220]. However, as with WGS-based serotyping, misclassification of atypical monophasic variants may also occur due to the intact specific PCR primer-binding sites of the *fljB* gene [211, 218]. Although the *fljB* gene may be intact, deletions or insertions within the phase 2 flagellar region may result in monophasic expression of atypical variants. Thus, *Salmonella* I 4,[5]12:i:- may be misclassified as genotypically biphasic when phenotypically it does not express the phase 2 flagerllar antigen and is monophasic.

2.5.4. Multilocus sequence typing

Multilocus sequencing typing (MLST) classifies *Salmonella* strains at a subserovar level by identifying evolutionary groups or clusters through their genetic relatedness. Consequently, MLST implements bacterial population genomics by inferring links between genotypes and phenotypic characteristics, such as the ability to cause disease. Although some clusters relate to one serovar, there are many clusters that include multiple serovars. Therefore, MLST does not differentiate serovars and should be performed in combination with traditional or WGS-based serotyping methods. *Salmonella enterica* sequence types (ST) are determined by using the internal fragments of seven house-keeping genes: *aroC, dnaN, hemD, hisD, purE, sucA,* and *thrA* [221]. The allele sequences of each internal fragment are determined and assigned an allele number. Together the seven numbers form the allelic profile of a strain and defines the respective sequence type. Two methods for MLST include PCR amplification of the house-keeping genes and *in silico*-based typing through WGS.

Currently, the PubMLST *Salmonella* Genome Databases website provides 6889 STs. The website uses the Bacterial Isolate Genome Sequence database (BIGSdb) genomic platform which links two distinct databases; the sequence definition database (contains the allelic profiles and identifiers) and the isolate/specimen database (provenance and epidemiological information) [222]. Furthermore, *Salmonella* STs that cluster together are further designated into eBurstGroups (eBGs) when six of the seven alleles that define the ST are shared or when there are ten or more ungrouped singleton STs [221]. In addition to the standard MLST scheme (seven locus), several other schemes

have been established. These schemes include core-genome MLST (cg-MLST), whole-genome MLST (wg-MLST), and ribosomal MLST (rMLST).

Although MLST does not differentiate between serovars, this method has been used to confirm relatedness of *Salmonella* I 4,[5],12:i:- to *Salmonella* Typhimurium. As seen in Table 2-1, the predominant STs of *Salmonella* I 4,[5],12:i:- are ST34 and ST19, both typical of *Salmonella* Typhimurium. ST34 is defined by the alleles aroC10-dnaN19-hemD12-hisD9-purE5-sucA9-thrA2 and ST19 only differs by dnaN7 [223]. In a previous study by Soyer et al., the majority of the *Salmonella* Typhimurium and *Salmonella* I 4,[5]12:i:- isolates were ST1 and a few U.S. *Salmonella* Typhimurium isolates were ST3 along with one *Salmonella* I 4,[5]12:i:- isolate from Spain. However, this study used a three-gene MLST scheme developed by Sukhnanand *et al* that included two different housekeeping genes (*manB, mdh*) and a virulence gene (*fimA*) [13, 224]. Furthermore, Achtman et al. discovered that most *Salmonella* Typhimurium belong to eBG1, but this group also contained various monophasic *Salmonella* I 4,[5],12:i:- strains. The presence of multiple monophasic variants within eBG1 suggests independent genetic emergence events, which has been supported by previous findings [17, 27, 72, 225]. These studies indicate *Salmonella* I 4,[5],12:i:- is genetically closely related to *Salmonella* Typhimurium [221]. Therefore, MLST is important subtyping tool in investigating phylogenetic evolutionary relationships and comparing population structure of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains.

Over the past years, there has been an increase in human foodborne illnesses related to antibiotic resistant *Salmonella* I 4,[5],12:i:- largely associated with the consumption of contaminated pork. Although several studies have established the genetic relationship between *Salmonella* Typhimurium and its monophasic variant, *Salmonella* I 4,[5], 12:i:-, the cause of increased incidence of salmonellosis attributed to this strain remains unknown. The differences in the phase 2 flagellar region of *Salmonella* I 4,[5],12:i:- clones worldwide, with similar phenotypic and genotypic AMR profiles, suggests *Salmonella* I 4,[5],12:i:- emerged through multiple independent events. The integration of the mobile genetic elements into this region indicates stabilization of new genetic material into the chromosome, allowing for clonal expansion. Furthermore, the reasons why *Salmonella* I 4,[5],12:i:- has established an ecological niche in swine is largely unknown and remains under investigation. Our study will allow us to identify the selection pressures and characteristics that have permitted *Salmonella* I 4,[5],12:i:- to emerge and expand in swine and their environment, leading to substantial cases of foodborne illness. *Salmonella* I 4,[5],12:i:- proves to be a food safety concern for the swine industry. Sound knowledge about *Salmonella* I 4,[5], 12:i:- and its potential virulence or competitive selection advantages is essential in developing mitigation strategies to eliminate this serovar on the farm or through processing to prevent dissemination into the food chain.

3. METHODS

3.1. USD-ARS-FFSRU Salmonella *isolates*

3.1.1. Subset collection of Salmonella isolates

A total of 125 *Salmonella* isolates were obtained from the United States Department of Agriculture – Agricultural Research Service – Food and Feed Safety Research Unit (USDA-ARS-FFSRU) College Station, TX (R. B. Harvey, NPB Grant #14-203). All *Salmonella* I 4,[5],12:i:- (n = 47) isolates were selected from the sample frame. For comparison, the following isolates also were selected based on serovars and phenotypic resistance profiles: *Salmonella* serovars Agona (n = 7), Alachua (n = 4), Derby (n = 9), Enteritidis (n = 8), Montevideo (n = 3), Senftenberg (n = 10), and Typhimurium (n = 37). Of these serovars, all except Typhimurium are classified as monophasic *Salmonella* (n = 88) while Typhimurium is a biphasic *Salmonella* serovar (n = 37).

For serovars Alachua, Enteritidis, Montevideo, and I 4,[5],12:i:-, all isolates were selected from the study by Harvey et al.; the number of isolates per serovar was 4, 8, 3, and 47, respectively. Alachua, Enteritidis, and Montevideo were selected for their monophasic characteristic. For serovars Agona, Derby, and Senftenberg, isolates were selected based on their resistance profile and also because they are monophasic *Salmonella*; the number of isolates selected per serovars was 7, 9, and 10, respectively; the total number of these serovars from the study by Harvey et al. were 12, 19, and 15, respectively. In addition, Typhimurium isolates were selected based on their resistance profiles and to compare this biphasic strain with I 4,[5],12:i:- and overall monophasic

strains. Typhimurium strains that had similar resistance profiles as I 4,[5],12:i:- isolates were selected; there were a total of 299 Typhimurium isolates from the study and 37 were selected for comparison.

3.1.2. Sample collection

The USDA-ARS-FFSRU collected cheek meat (CM) and head trim (HT) from swine carcasses at a large commercial pork processing plant in the southern United States, which serviced several grower farms in the United States Midwest. Sampling over a two-day period occurred every other month over 12 months from January to December (A-F), 2015. Each day, 25 samples of each tissue (CM and HT) were collected from an equal number of slaughter hogs in the morning and another 25 in the afternoon. During a two-day period, a total of 100 CM and 100 HT tissue samples were collected, totaling 200 samples per month. The total number of samples collected over the study was 1200: 600 CM and 600 HT. The sampling dates and times were determined in advance for equal representation of sow herds and geographical locations of those regional farms producing market hogs.

3.1.3. Salmonella isolation from tissue samples

Both CM and HT tissue samples were processed by the USDA-ARS as previously described [226]. Tissues were weighed then briefly submerged in boiling water for 3-5 seconds to sterilize the surface. These parboiled tissues were placed into sterile filtered-stomacher bags (Nasco, Fort Atkinson, WI) and pulverized using a rubber mallet. Then, 80 ml of tryptic soy broth (TSB) (Becton Dickinson, Sparks, MD) was added, and the

tissue was homogenized for 30 seconds with a laboratory blender (BagMixer 400VW, Interscience Laboratories Inc., Weymouth, MA). After homogenization, the samples were non-selectively enriched (pre-enriched homogenate) for bacterial growth by first placing them in the incubator for 2 hours at 25°C, then incubating for 12 hours at 42°C, and finally storing in a refrigerator at 4℃ until further processing.

Two different methods were used by the USDA-ARS-FFSRU to isolate *Salmonella*. The first method was performed immediately after the homogenization and prior to incubation by plating 1 ml of the homogenate onto 3M Petrifilm™ Enterobacteriaceae (EB) Count Plates (3M Microbiology, St. Paul, MN), in duplicate, following the manufacturer's instructions. The EB Count Plates were incubated for 18-22 h at 37°C. After incubation, gas-producing colonies were identified and counted before storing the EB Count Plates at 4°C. Gas-producing colonies were defined as red colonies associated with gas bubbles or red colonies associated with yellow zones and gas bubbles. Plates were stored until results from the enrichment process were obtained. The second method, a specific enrichment culture method, started by subjecting 1 ml of the pre-enriched homogenate to anti-*Salmonella* immunomagnetic separation (IMS). Twenty µl of anti-*Salmonella* beads (Invitrogen, Carlsbad, CA) were added to each 1 ml of homogenate then incubated for 15 min at room temperature (RT). After incubation, the beads were removed and double-washed with PBS-Tween 20. The washed beads were placed in 3 ml of Rappaport Vassiliadis soya peptone broth (RVS) (Remel Products, Lenexa, KS) and incubated overnight at 42°C. For *Salmonella* detection, the RVS culture

was swabbed and streaked onto Brilliant Green Agar (BGA) (BD, Sparks, MD) with sulfadiazine (80 mg/l) (Sigma, St. Louis, MO). BGA plates were incubated for 18-20 h at 37°C; thereafter, up to three suspected *Salmonella* colonies were selected for confirmation. If tissue samples were positive for *Salmonella* via enrichment, EB Count Plates were replica plated onto xylose lysine deoxycholate agar (XLD) plates. The XLD plates were incubated for 18-20 h at 37°C and three suspected *Salmonella* colonies were selected for confirmation.

Suspected *Salmonella* colonies selected from the XLD and BGA plates were streaked onto BD Trypticase™ Soy Agar II (TSA) with 5% sheep blood. The presumptive *Salmonella* were confirmed via triple sugar iron agar (TSIA) and lysine iron agar (LIA) slants. After confirmation, the *Salmonella* were preserved in CryoBeads™ (ThermoFisher Scientific, Waltham, MA) and stored at -80°C for future use.

3.1.4. White-Kauffmann-Le Minor scheme: traditional Salmonella serotyping

Traditional slide agglutination, also referred as O typing, was performed by the USDA-ARS-FFSRU (College Station, TX). Following the manufacturer's instructions, individual BD Difco *Salmonella* O antisera (Difco, Sparks, MD) were used to determine the specific serogroup of each isolate. A selection of isolates was sent to the USDA-Animal and Plant Inspection Services, National Veterinary Services Laboratory, Ames, IA, for traditional *Salmonella* serotyping using the White-Kauffmann-Le Minor scheme.

3.2. Phenotypic Analysis

3.2.1. Antimicrobial susceptibility testing

The Sensititre™ National Antimicrobial Resistance Monitoring System (NARMS) CMV3AGNF custom plate (TREK Diagnostics Inc., Cleveland, OH) was used by the USDA-ARS-FFSRU to assess the **s**usceptibility of *Salmonella* isolates to 14 antimicrobial agents and determine the minimum inhibitory concentration (MIC) (Figure 5-1). The automated broth microdilution method was performed via the Sensititre™ system (TREK Diagnostics Inc., Cleveland, OH) according to the manufacturer's guidelines. Briefly, isolates preserved in CryoBeads™ were streaked onto TSA with 5% sheep blood and incubated for 18 h at 37°C. After incubation, one or two colonies were inoculated into 5 ml of sterile demineralized water and were standardized using the 0.5 Polymer McFarland Standard and Sensititre™ Nephelometer (TREK Diagnostics Inc., Cleveland, OH). Ten µl of the standardized culture was added to 11 ml of BBL™ Mueller Hinton II Broth (Cation-Adjusted) (MHB) (TREK Diagnostics Inc., Cleveland, OH). Using the Sensititre AIM™ Automated Inoculation Delivery System (TREK Diagnostics Inc., Cleveland, OH), 50 µl of the suspension was inoculated into each well of the CMV3AGNF plate. The plates were incubated for 18 h at 37°C then read using the Sensititre OptiRead™ Automated Fluorometric Plate Reading System (TREK Diagnostics Inc., Cleveland, OH). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213 (American Type Culture Collection, Manassas, VA) were used as quality control strains for susceptibility testing.

The NARMS plate contained antimicrobials in 9 different antibiotic classes including: penicillins, beta-lactam combinations, cephems, aminoglycosides, macrolides, tetracyclines, quinolones/fluoroquinolones, folate pathway antagonists, and phenicols (Figure 3-1). The USDA-ARS-FFSRU provided the raw microbroth dilution data; subsequently, data analyses of the isolates were performed by our laboratory using the most updated interpreted clinical breakpoints. Isolates were classified as susceptible, intermediate, or resistant as defined by the Clinical and Laboratory Standards Institute (CLSI, 2020), when available; otherwise, NARMS consensus breakpoints were used. *Salmonella* isolates were categorized as multidrug-resistant (MDR) when resistant to three or more antimicrobial classes and decreased susceptibility to ciprofloxacin (DSC, MIC ≥ 0.12 μg/ml) as defined by NARMS. In addition, maximum MIC tested for sulfisoxazole is 256 μg/ml, all isolates harboring the *sul* gene(s) and with a MIC of 256 μg/ml were classified as resistant to sulfisoxazole.

SENSITITRE GRAM NEGATIVE NARMS PLATE FORMAT

Plate code: CMV3AGNF

	1	2	3	4	5	6	7	8	9	10	11	12
A	FOX	AZI	CHL	AXO	AXO	CIP	GEN	NAL	XNL	FIS	AMP	STR
	32	8	16	64	0.25	2	16	16	2	32	32	16
B	FOX	AZI	CHL	AXO	AUG2	CIP	GEN	NAL	XNL	FIS	AMP	STR
	16	4	8	32	32/16	1	8	8	1	16	16	8
C	FOX	AZI	CHL	AXO	AUG2	CIP	GEN	NAL	XNL	SXT	AMP	STR
	8	2	4	16	16/8	0.5	4	4	0.5	Apr-76	8	4
D	FOX	AZI	CHL	AXO	AUG2	CIP	GEN	NAL	XNL	SXT	AMP	STR
	4	1	2	8	8/4	0.25	2	2	0.25	Feb-38	4	2
E	FOX	AZI	TET	AXO	AUG2	CIP	GEN	NAL	XNL	SXT	AMP	NEG
	2	0.5	32	4	4/2	0.12	1	1	0.12	19-Jan	2	
F	FOX	AZI	TET	AXO	AUG2	CIP	GEN	NAL	FIS	SXT	AMP	POS
	1	0.25	16	2	2/1	0.06	0.5	0.5	256	0.5/9.5	1	
G	FOX	AZI	TET	AXO	AUG2	CIP	GEN	XNL	FIS	SXT	STR	POS
	0.5	0.12	8	1	1/0.5	0.03	0.25	8	128	0.25/4.75	64	
H	AZI	CHL	TET	AXO	CIP	CIP	NAL	XNL	FIS	SXT	STR	POS
	16	32	4	0.5	4	0.015	32	4	64	0.12/2.38	32	

	ANTIMICROBICS
FOX	Cefoxitin
AZI	Azithromycin
CHL	Chloramphenicol
TET	Tetracycline
AXO	Ceftriaxone
AUG2	Amoxicillin/clavulanic acid 2:1 ratio
CIP	Ciprofloxacin
GEN	Gentamicin
NAL	Naladixic Acid
XNL	Cetiofur
FIS	Sulfisoxazole
SXT	Trimethoprim/sulfamethoxazole
AMP	Ampicillin
STR	Streptomycin
NEG	Negative Control
POS	Positive Control

Figure 3-1 Sensititre Gram Negative NARMS CMV3AGNF Plate Format

The *Salmonella* I 4,[5],12:i:- (n = 47) and *Salmonella* Typhimurium (n = 37) isolates were further characterized using the Sensititre™ Vet Bovine/Porcine BOPO6F plate (TREK Diagnostics Inc., Cleveland, OH) to determine the MIC for enrofloxacin (Figure 3-2). The same procedure as stated above was followed for the BOPO6F plate. The breakpoint for enrofloxacin was determined by the CLSI breakpoint (CLSI, 2020) established for *Enterobacteriaceae* isolated from dogs since there is no MIC established for *Enterobacteriaceae* or *Salmonella* isolated from humans or swine. However, isolates can only be considered intermediate to enrofloxacin as the maximum concentration of enrofloxacin used in the BOPO6F plate was 2 µg/ml. The MIC for enrofloxacin was used for the bacterial fitness growth curve study. All breakpoints used in this study are found in Table 3-1.

SENSITITRE BOVINE/PORCINE PLATE FORMAT

Plate code: BOPO6F

	1	2	3	4	5	6	7	8	9	10	11	12
A	XNL	TIA	CTET	OXY	PEN	AMP	DANO	SXT	TYLT	TUL	CLI	SDM
	8	32	8	8	8	16	1	Feb-38	4	4	16	256
B	XNL	TIA	CTET	OXY	PEN	AMP	DANO	SPE	TYLT	TUL	CLI	ENRO
	4	16	4	4	4	8	0.5	64	2	2	8	2
C	XNL	TIA	CTET	OXY	PEN	AMP	DANO	SPE	TYLT	TUL	CLI	ENRO
	2	8	2	2	2	4	0.25	32	1	1	4	1
D	XNL	TIA	CTET	OXY	PEN	AMP	DANO	SPE	TYLT	TUL	CLI	ENRO
	1	4	1	1	1	2	0.12	16	0.5	64	2	0.5
E	XNL	TIA	CTET	OXY	PEN	AMP	NEO	SPE	TUL	TIL	CLI	ENRO
	0.5	2	0.5	0.5	0.5	1	32	8	64	32	1	0.25
F	XNL	TIA	TIA	GEN	PEN	AMP	NEO	TYLT	TUL	TIL	CLI	ENRO
	0.25	1	0.5	0.25	0.25	0.5	16	32	32	16	0.5	0.12
G	GEN	GEN	GEN	GEN	PEN	AMP	NEO	TYLT	TUL	TIL	CLI	POS
	8	4	2	0.12	0,25	0.25	8	16	16	8	0.25	
H	FFN	FFN	FFN	FFN	FFN	FFN	NEO	TYLT	TUL	TIL	POS	POS
	8	4	2	0.5	0.25	0.25	4	8	8	4		

	ANTIMICROBICS
XNL	Ceftiofur
TIA	Tiamulin
CTET	Chlortetracycline
GEN	Gentamicin
FFN	Florfenicol
OXY	Oxytetracycline
PEN	Penicillin
AMP	Ampicillin
DANO	Danofloxacin
SDM	Sulphadimethoxine
NEO	Neomycin
SXT	Trimethoprim/sulfamethoxazole
SPE	Specttinomycin
TYLT	Tylosin tartrate
TUL	Tulathromycin
TIL	Tilmicosin
CLI	Clindamycin
ENRO	Enrofloxacin
POS	Positive Control

Figure 3-2 Sensititre Vet Bovine/Porcine BOPO6F Plate Format

Table 3-1 Range and MIC breakpoints used for *Salmonella* spp.

ANTIMICROBIAL CLASS		ANTIMICROBIAL AGENT	RANGE	MIC BREAKPOINTS (µg/ml)		
			(µg/ml)	**Susceptible (S)**	**Intermediate (I)**	**Resistant (R)**
B-LACTAMS	**PENICILLINS**	Ampicillin (A)[1]	1-32	≤ 8	16	≥ 32
	B-LACTAM COMBINATIONS	Amoxicillin/clavulanic acid (Au)[1]	1/0.5-32/16	≤ 8/4	16/8	≥ 32/16
	CEPHEMS	Cefoxitin (Cn)[1]	0.5-32	≤ 8	16	≥ 32
		Ceftriaxone (Cx)[1]	0.25-64	≤ 1	2	≥ 4
		Ceftiofur (Cr)[2]	0.12-8	≤ 2	4	≥ 8
AMINOGLYCOSIDES		Gentamicin (G)[1]	0.25-16	≤ 4	8	≥ 16
		Streptomycin (S)[3]	2-64	≤ 16	-	≥ 32
MACROLIDES		Azithromycin (Az)[1]	0.12-16	≤ 16	-	≥ 32
TETRACYCLINES		Tetracycline (T)[1]	4-32	≤ 4	8	≥ 16
QUINOLONES AND FLUOROQUINOLONES		Ciprofloxacin (Cp)[1]	0.015-4	≤ 0.06	0.12-0.5	≥ 1
		Enrofloxacin (E)[4]	-	≤ 0.5	1-2	≥ 4
		Nalidixic acid (Na)[1]	0.5-32	≤ 16	-	≥ 32
FOLATE PATHWAY ANTAGONISTS		Trimethoprim-Sulfamethoxazole (SxT)[1]	0.12/2.4-4-76	≤ 2/38	-	≥ 4/76
		Sulfisoxazole (Su)[1]	16-256	≤ 256	-	≥ 512
PHENICOLS		Chloramphenicol (C)[1]	2-32	≤ 8	16	≥ 32

[1]CLSI M100 ED30:2020, clinical breakpoints
[2]CLSI VET08 ED4:2018, Ceftiofur breakpoint for *Salmonella* isolated from swine
[3]NARMS-established breakpoints for resistance monitoring
[4]CLSI VET08 ED4:2018, Enrofloxacin breakpoint for *Enterobacteriaceae* isolated from dogs

3.2.2. Bacterial fitness growth curves

The fitness of *Salmonella* strains was assessed using the Bioscreen C™ Automated Microbiology Growth Curve Analysis System (Bioscreen C) (Growth Curves USA, Piscataway, NJ). Using the Bioscreen C, we compared the fitness of *Salmonella* I 4,[5],12:i:- strains to *Salmonella* Typhimurium strains, as well as monophasic to biphasic strains under varying selection pressures and evaluated the fitness cost of important genes associated with antimicrobial resistance (AMR). The *Salmonella* strains were subjected to different concentrations of ceftiofur (2, 4, 6, and 8 µg/ml), enrofloxacin (0.25, 0.5, 1, 2, and 4 µg/ml), and tetracycline (4, 6, 8, 12, 14, and 16 µg/ml) between the susceptible and resistant CLSI MIC breakpoints [227]. Bacterial growth curves were determined by measuring the turbidity of the growth medium - optical density (OD) - for a specified time period.

Salmonella isolates were streaked onto TSA with 5% sheep blood and incubated for 18 h at 37°C. Two ATCC strains, *Escherichia coli* ATCC 32518 and *Pseudomonas aerugenosa* ATCC 27853, were also streaked; these strains served as positive controls and plain MHB served as a negative control. Following incubation, one or two colonies were inoculated into 5 ml of sterile demineralized water. The turbidity of the samples were adjusted to the 0.5 Polymer McFarland Standard using the Sensititre™ Nephelometer (TREK Diagnostics Inc., Cleveland, OH). Then, 30 µl of the standardized isolates was inoculated in triplicate into the wells of the Bioscreen C honeycomb plates (manufacturer) (Figure 3-3) containing 270 µl of MHB medium prepared with the appropriate antibiotic

and concentration in each well. The Bioscreen C experiment was set for 48 h at 37°C. The Bioscreen C measured the turbidity of the inoculum and medium mixture in the honeycomb plates every 10 min at $OD_{420\text{-}500}$; before measurement, the plates were lightly shaken for 10 sec to ensure uniformity. For a baseline growth curve, all *Salmonella* strains were inoculated and grown in plain MHB.

Microplate number 1

1	11	21	31	41	51	61	71	81	91
2	12	22	32	42	52	62	72	82	92
3	13	23	33	43	53	63	73	83	93
4	14	24	34	44	54	64	74	84	94
5	15	25	35	45	55	65	75	85	95
6	16	26	36	46	56	66	76	86	96
7	17	27	37	47	57	67	77	87	97
8	18	28	38	48	58	68	78	88	98
9	19	29	39	49	59	69	79	89	99
10	20	30	40	50	60	70	80	90	100

Microplate number 2

101	111	121	131	141	151	161	171	181	191
102	112	122	132	142	152	162	172	182	192
103	113	123	133	143	153	163	173	183	193
104	114	124	134	144	154	164	174	184	194
105	115	125	135	145	155	165	175	185	195
106	116	126	136	146	156	166	176	186	196
107	117	127	137	147	157	167	177	187	197
108	118	128	138	148	158	168	178	188	198
109	119	129	139	149	159	169	179	189	199
110	120	130	140	150	160	170	180	190	200

Figure 3-3 Honeycomb microplate layout

3.2.2.1. Data Analysis and Statistics

Turbidity measurement results were collected and analyzed using a 3-parameter Gompertz model, preprogrammed as a nonlinear regression module, in Stata® ver. 15.1 (StataCorp LLC, College Station, TX). Along with generating the 3-parameter Gompertz-model for each Bioscreen C run at each specific concentration of an antibiotic, including the baseline, graphical presentation of the fitted growth curves was performed in Stata® ver. 15.1. The 3-parameter Gompertz function is as follows (Equation 3-1):

$$OD = b_1 * exp(-exp(-b_2 * (Time - b_3)))$$

Equation 3-1 3-parameter Gompertz model

where OD is the dependent variable representing the optical density and Time is the independent variable. The three parameters are b_1, b_2, and b_3 which represent the stationary phase, exponential phase (also known as the maximum growth rate), and lag phase, respectively. The 3-parameter Gompertz model allows for an asymmetrical monophasic sigmoidal curve and is commonly used to interpret bacterial growth curves (Figure 3-4) [228, 229].

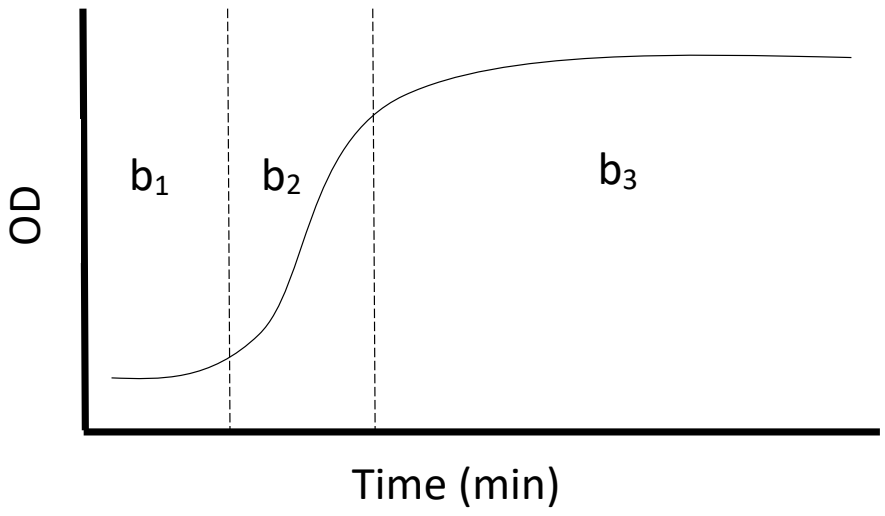


Figure 3-4 Nonlinear regression: 3-parameter Gompertz model

Growth curves for *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium, as well as monophasic and biphasic strains at varying concentrations and different antibiotics,

were generated for comparison. Using whole-genome sequencing data, we evaluated the fitness cost of several antimicrobial resistance (AMR) genes including, *bla*$_{\text{SHV-12}}$ (ceftiofur), *qnrB* (enrofloxacin), and *tet* (tetracycline) by generating fitted growth curves for *Salmonella* I 4,[5],12:i:-, *Salmonella* Typhimurium, and monophasic strains with the presence of each AMR gene (3.4 Genotypic analysis). To interpret whether there were significant differences between the curves, we compared each of the 95% confidence intervals (95% CI) from the three individual parameters from each model generated. If there was overlap of 95% CI, there was no significant difference at $p > 0.05$. If there was no overlap of 95% CIs, there was a significant difference at $p \leq 0.05$. Additionally, when the Gompertz model did not converge, no curve was generated.

3.2.3. Motility assays

In vitro motility assays were performed to estimate and compare swimming and swarming differences between biphasic *Salmonella* Typhimurium and monophasic *Salmonella* I 4,[5],12:i:-, as well as between biphasic and monophasic *Salmonella* strains overall, using modifications to methods previously described [230]. *Salmonella* isolates were streaked onto TSA with 5% sheep blood and incubated for 18 h at 37°C, including *Salmonella enterica* ATCC 700720 as a positive control. One colony was inoculated in 5 ml of Luria-Bertani broth (LBB) (Difco, Sparks, MD) and incubated for 16 h at 37°C. The overnight bacterial culture was diluted 1:10 in LBB by adding 20 µl of culture to 180 µl of LBB in a honeycomb plate. The Bioscreen C was used to incubate diluted cultures for 2 h at 37°C to reach an OD_{600} of approximately 0.3.

Swim and swarm assays were differentiated by the percentage of agar and addition of glucose. Swim assays were prepared with 0.3% agar and nutrient broth (NB) (NB no.2 Oxoid, ThermoFisher Scientific, Waltham, MA); whereas, swarm assays were prepared with 0.5% agar, NB and 0.5% glucose [w/v]. A total of 25 ml of either swim or swarm media was dispensed in sterile petri dishes and dried overnight for 24 hours at RT with the petri dish lids on. After incubation of the diluted culture in 1:10 LBB and following the 24 h drying period, 10 µl of the bacterial culture was spotted at the center of each motility assay agar plate and then incubated for 18 h at 37°C with the petri dishes placed upright. The diameter of the halo, which represents the movement of *Salmonella*, was measured in mm at 6, 12, and 18 hours for both swim and swarm assays. The petri dish is 88 mm in diameter; maximum growth was capped at 85 mm. The motility assays were performed in triplicate for each isolate and the final results were the mean diameter of the three technical triplicates.

3.2.3.1. Data Analysis and Statistics

The Student's t-test was performed to determine significant differences in swimming and swarming motility between the following groups: monophasic and biphasic *Salmonella* isolates, multidrug-resistant (resistant to 3 or more antibiotic classes) and non-MDR (pan-susceptible or resistant to 2 or fewer antibiotic classes) *Salmonella* isolates, and *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates. A one-way analysis of variance (ANOVA) was performed to determine if there was a statistically significant difference in between the mean of three or more serovars in swimming and

swarming ability. If there were differences between the means, the Bonferroni multi-comparisons test was used to determine differences between swimming and swarming between of all serotypes in the study. A *p*-value less than 0.05 ($p \leq 0.05$) is statistically significant. The statistical analyses were performed using Stata® v15.

3.2.4. Biofilm assays

Biofilm formation was assessed using the 96-well microtiter dish biofilm formation assay method as previously described (with minor modifications) [203]. This method included a series of steps such as growing, fixing, staining, and quantifying the biofilm to classify the biofilm producers. Briefly, *Salmonella* isolates were streaked onto TSA with 5% sheep blood and incubated for 18 hours at 37°C, including *Salmonella enterica* ATCC 700720 as a positive control. One colony was inoculated into 1 ml of TSB and incubated for 24 h at 37°C. A sterile 96-well flat-bottomed polystyrene tissue-culture treated microplate (96-well plate) (VWR, Radnor, PA) was filled with 230 µl of 1:10 TSB (nutrient-limiting media) along with 20 µl of the TSB bacterial culture and incubated for 24 h at 37°C to grow the biofilm; the negative control contained only broth. After incubation, planktonic cells were removed gently by aspiration so as to not disrupt the biofilm and the microplate was air-dried for 20 min. The biofilms were washed three times with 300 µl of sterile demineralized water and air-dried for 20 min. A total of 250 µl of methanol was added to each well to fix the biofilms and incubated for 15 min at RT and then removed. The biofilms were stained with 125 µl of 0.1% crystal violet and incubated for 15 min at RT. The crystal violet was removed, and the wells rinsed with sterile

demineralized water until the water was clear. The microplates were air-dried for another 20 min. To resolubilize the biofilms, 200 µl of 95% ethanol was added and the plate was incubated for 15 min at RT. The biofilms were resuspended until homogeneous and 125 µl of the resolubilized mixture was transferred to a new 96-well plate. The OD of each well containing the biofilm was measured at 600 nm using the FLUOStar Omega Microplate Reader (BMG LABTECH, Cary, NC) for quantification.

3.2.4.1. Data Analysis and Statistics

Biofilm assays were performed in biological and technical triplicate and the results were averaged. Based on the OD_{600} readings, the *Salmonella* strains were classified into four different categories: no, weak, moderate, or strong biofilm producers, as previously described [231]. The cut-off OD (OD_c) is defined as three standard deviations above the mean OD of the negative control. The classification is as follows: $OD \leq OD_c$ = no biofilm producer, $OD_c < OD \leq (2 \times OD_c)$ = weak biofilm producer, $(2 \times OD_c) < OD \leq (4 \times OD_c)$ = moderate biofilm producer, $(4 \times OD_c) < OD$ = strong biofilm producer. The differences in biofilm formation between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium were assessed by the two-sample Wilcoxon-Mann-Whitney signed ranks-sum test (Mann-Whitney) using Stata® v15. The Kruskal-Wallis equality-of-populations rank test (Kruskal-Wallis) was used to determine if there are statistical differences in biofilm production between *Salmonella* serovars followed by the Mann-Whitney test. Differences were significant when the *P*-value ≤ 0.05. Additionally, a linear mixed model was used to

account for biofilm-related genes (3.3 Genotypic analysis) and phase characteristic between *Salmonella* serovars. Replicates was included as a random effect.

3.3. Genotypic analysis

3.3.1. Whole-genome sequencing of isolates

Whole genome sequencing (WGS) of *Salmonella* isolates was performed to conduct genome analyses including determining the genotypic serotype, multilocus sequence type (MLST), *Salmonella* Pathogenicity Islands (SPIs), as well as the presence of AMR genes, DNA gyrase and topoisomerase IV point mutations, plasmids, flagellar genes, and biofilm-related genes. Genome alignment, SNP analysis, and phylogenetic analyses of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium were also conducted with the generated sequences. Short- and long-read sequences was used to complete and close the genome of five *Salmonella* I 4,[5],12:i:- isolates.

3.3.1.1. DNA extraction

Salmonella DNA was extracted using the QIAmp 96 DNA QIAcube HT Kits and the QIAcube HT instrument (both: Qiagen, Valencia, CA) as previously described [130]. Briefly, *Salmonella* isolates were streaked onto TSA with 5% sheep blood and incubated overnight at 37°C. One colony was suspended into 5 ml of TSB and incubated overnight at 37°C. A 1 ml aliquot of the overnight culture was transferred into a 1.2 ml micro-collection tube, centrifuged for 15 min at 4,000 rpm followed by removal of the supernatant. The remaining pellet was resuspended in 200 µl of ATL buffer and DX reagent mixture (Qiagen). For mechanical lysis of the bacterial cells, one tube of small pathogen lysis beads (Qiagen) was added to the suspension then disrupted with the TissueLyser system (Qiagen) for 5 min at 25 Hz. The tubes were centrifuged briefly to remove the foam and to settle the beads. For protein digestion, 40 µl of Proteinase K was added to each tube. The tubes were incubated for 1 h at 56°C at 900 rpm then heat shocked for 10 min at 95°C

in a ThermoMixer (Eppendorf, Hauppauge, NY). Following incubation, the suspension was cooled to RT. For RNA digestion, 4 µl of RNAse A was added and incubated for 5 min at RT. The prepared lysates were set in the QIAcube HT for DNA isolation using a modified QIAamp® 96 DNA QIAcube® HT Protocol [130].

3.3.1.2. DNA quality/quantity

The quality and quantity of the DNA were evaluated using the FLUOStar Omega Microplate Reader and Qubit 3 Fluorometer (ThermoFisher). DNA quality was determined by the 260/280 ratio. When 260/280 ratios are less than 1.8 (presence of proteins) or greater than 2.0 (presence of RNA), the DNA was purified using the Zymo Research DNA Clean & Concentrator Kit (Genesee Scientific, San Diego, CA). For short-read sequencing, DNA quantity was measured with the Quant-iT™ Pico Green® dsDNA Assay kit (ThermoFisher) and FLUOstar Omega. For long-read sequencing, DNA quantity was measured with the Qubit™ dsDNA High Sensitivity Assay Kits (ThermoFisher) and Qubit. The DNA was stored at -30°C until further use.

3.3.1.3. Whole-genome sequencing by Illumina MiSeq Platform

All *Salmonella* isolates (n = 127) were sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA, USA), a short-read sequencing technology. Whole-genome sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) with a fragment length of 600 bp and run with the MiSeq Reagent Kit v3 with 2 × 300-bp paired-end reads. Standard Illumina protocols were followed for library preparation (Figure 3-5).

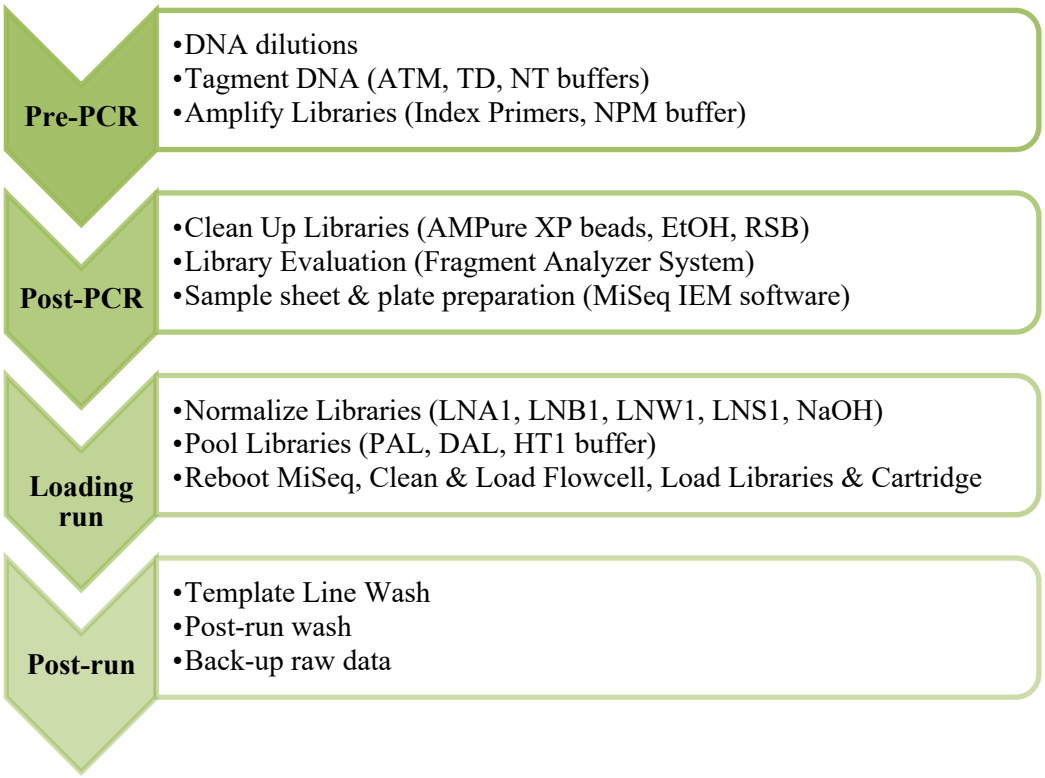


Figure 3-5 Illumina MiSeq Library Preparation Workflow (Nextera XT)

3.3.1.4. Whole-genome sequencing by Oxford Nanopore MinION platform

Five *Salmonella* I 4,[5],12:i:- isolates was selected based on their antimicrobial resistance profiles for sequencing on the Oxford Nanopore Technologies MinION (Oxford Nanopore Technologies, Oxford, UK) platform, a long-read sequencing technology. The Rapid Barcoding Sequencing kit (SQK-RBK004, Oxford Nanopore Technologies, Oxford, UK) was used to multiplex and prepare sequencing libraries for the selected *Salmonella* I 4,[5],12:i:- DNA, according to the manufacturer's protocol. Briefly, 400 ng of input genomic DNA (gDNA) was prepared in nuclease-free water (NFW) (ThermoFisher Scientific, Waltham, MA); the total volume was then adjusted to 7.5 µl. For each gDNA sample, 2.5 µl of the fragmentation mix RB01-05

(one per sample) was added to fragment and barcode the DNA. The mixture was incubated for 1 min at 30°C, 1 min at 80°C, and then cooled to RT. All barcoded samples were then pooled, noting the total volume. An equal amount of resuspended AMPure XP beads was added and incubated for 5 min at RT on a rotator mixer. The sample was spun down, pelleted on a magnet, and the supernatant removed. Keeping the tubes on the magnet, the beads were washed twice with 200 µl of 70% ethanol. After the last wash, all residual 70% ethanol was removed, and the beads were air-dried for approximately 10 min. The tube was then removed from the magnet rack to resuspend the pellet in 10 µl of 10 mM Tris-HCl (pH 7.5-8.0) with 50 mM NaCl (Sigma-Aldrich Co LLC, Merck KGaA, Darmstadt, Germany); next, the resuspension was incubated for 2 min at RT. Once again, the beads were pelleted on a magnet until the eluate was clear. Ten µl of eluate containing the DNA was removed and added to a clean 1.5 ml Eppendorf DNA LoBind tube. One µl of rapid adapter (RAP) was added to the 10 µl barcoded DNA and incubated for 5 min at RT. Finally, the prepared library was loaded onto the MinION flow cell (R4.9.1) and placed into the MinION (Oxford Nanopore Technologies, Oxford, UK) to start the sequencing run (Figure 3-6).

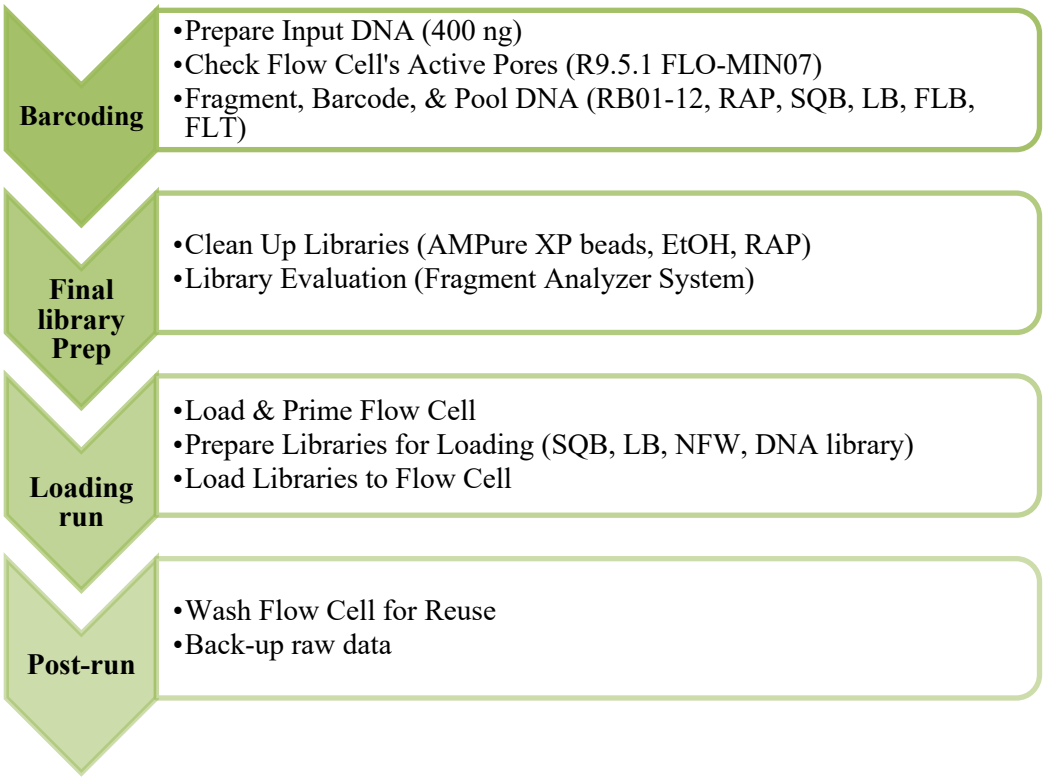


Figure 3-6 Oxford Nanopore Technologies MinION Library Preparation Workflow (SQK-RBK004)

3.3.2. Bioinformatics

Default parameters were used for all bioinformatics software unless otherwise specified. FASTQ files containing the raw reads were produced after short-read and long-read sequencing runs using the Illumina MiSeq and Oxford Nanopore Technologies MinION, respectively.

3.3.2.1. De novo assembly

FastQC v0.11.4 was used to assess the quality of all raw reads and to determine the total number of reads generated. For MiSeq short-reads, Trimmomatic v0.38 was used to remove adapter/primer sequences, low-quality bases and reads with a quality threshold of 3 for reading

and trailing and the genomes were assembled using the *de novo* assembler SPAdes v3.11.1. For MinION long-reads, Guppy v2.3.5 was used to remove barcodes and reads that contain the internal barcode adapter sequence, as well as remove reads shorter than 500 bp or having an average quality score < 9. See section *Complete genome assembly* for hybrid assembly (3.4.2.2) of Miseq and MinION reads. The contiguity and quality of all assemblies was evaluated using QUAST v3.2.

3.3.2.2. Complete genome assembly

Using previously assembled MiSeq short-reads, two hybrid assembly methods were used to achieve high-quality closed genomes or the most complete genome: MiSeq assemblies with demultiplexed MinION reads and MiSeq assemblies with MinION assemblies. For the first method, the Unicycler v0.4.0 assembly tool was used to assemble demultiplexed MinION long-reads with MiSeq assemblies. In the second method, the Canu v1.8 assembly tool that specializes in assembly of long-read sequences was used to assemble MinION reads. After the long-read assembly, BWA v0.7.15 was used to align the MinION assemblies and MiSeq assemblies. The final hybrid assembly of the second methods was polished using Pilon v1.22. Benchmarking Universal Single-Copy Orthologs (BUSCO) was used to analyze the genome assemblies for completeness. The goal was to complete the genome to improve coverage, reduce error rate, and obtain one contig; however, the assembly with the smallest number of contigs and best coverage depth was selected for further analysis. SnapGene Viewer v5.3 was used to visualize and detect the location of mobile genetic elements along with resistance or virulence genes located within the flagellar phase 2 region.

3.3.2.3. Genotypic serotype, MLST, AMR genes, point mutations, and plasmids

The Center for Genomic Epidemiology (CGE) web-interface tools were used for analysis of the *Salmonella* raw sequences obtained from the Illumina MiSeq. The genotypic serotype and MLST were determined using SeqSero v1.2 and MLST v2.0, respectively [215, 232]. ResFinder v3.1.0 was used to detect AMR genes and PointFinder v3.1.0 to detect chromosomal point mutations [233]. The most up-to-date databases at the time of analysis were used for each of the tools.

3.3.2.4. Biofilm-related genes and heavy metal resistance genes

The presence of biofilm-related genes was identified and used to compare the ability of the *Salmonella* strains to form a biofilm. Biofilm-related genes include, but are not limited to, *adrA*, *fimH*, *csg*A, *csgB*, *csgD*, *sirA*, *glyA*, *ompR*, *sdiA*, *sipB*, *sipC*, *luxS*, *pfs*, and *gcpA*. In addition, heavy metal tolerance (HMT) genes for copper (*pcoABCDRSE*), silver (*silSECBAP*) and mercury (*merACDEPTR*) were identified. To identify the biofilm-related genes, PATRIC v3.6.2 Genome Annotation Service was used to provide annotations of assembled *Salmonella* FASTA files. The Genome Annotation Service uses the RAST tool kit (RASTtk) for annotation of genomic features. The HMT genes were identified using the FASTA files in ABRicate v. 0.9.9 against the MEGARes 2.0 database to screen contigs for HMT genes. [234, 235].

3.3.2.5. Mapping of Salmonella I 4,[5],12:i:-

All *Salmonella* I 4,[5],12:i:- strains were mapped to the *Salmonella* Typhimurium str. LT2 reference genome (NC_003197) to evaluate and observe deletions and/or mutations in the phase-2 flagellum locus region. Geneious Prime v2019.2.1 software was used to map the *Salmonella* I 4,[5],12:i:- FASTQ forward and reverse MiSeq sequences to the annotated *Salmonella*

Typhimurium GenBank file of NC_003197. Genes of interest were *flj*B, *flj*A, *hin*, and *iroB,* which encode for the phase-2 flagella, repressor of *fliC* gene that encodes for phase-1 flagella, the Hin recombinase, and putative iron-related glycosyltransferase, respectively. Furthermore, SnapGene Viewer v5.2.4 was used to visualize the phase 2 flagellar antigen region.

3.4. Evolutionary analysis

Default parameters were used for all bioinformatics software unless otherwise specified. The complete genome assembly of *Salmonella* Typhimurium str. LT2 reference strain (NCBI accession number NC_003197) was used as a reference for the tree analysis. Three phylogenetic trees were generated: (1) *Salmonella* I 4,[5],12:i:- (n = 46) and *Salmonella* Typhimurium (n = 38) genomes from our study, (2) *Salmonella* I 4,[5],12:i:- from our study (n = 46) and publicly available *Salmonella* I 4,[5],12:i:- genomes (n = 655), and (3) all *Salmonella* I 4,[5],12:i:- (n = 701) as well as *Salmonella* Typhimurium (n = 38) genomes from our study and publicly available *Salmonella* Typhimurium (n = 208) genomes. There were a total of 701 *Salmonella* I 4,[5],12:i:- and 246 *Salmonella* Typhimurium genomes included in the analyses, totaling to 947 genomes.

3.4.1. Selection of publicly available genomes on EnteroBase

Publicly available *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes available on NCBI were selected using EnteroBase; strains were filtered under the 'Strain Metadata' tab by serovar, continent, and source type and selected based on the Hierarchical Clustering of Core-genome MLST (cgMLST) (HeirCC) [236]. SeqSero2 serovar determination was used to select serovars. For the monophasic *Salmonella* Typhimurium variant, the serovar search included 1,4,[5],12:I:-, 4,[5],12:i:-, 4,[5],12: I:-, 4,[5],12:I-, 4,[5],12:I:-, 1,4,[5],12:I:-,

1,4,[5],12 : I : -, and 4,5,12:i:-. As for *Salmonella* Typhimurium, the serovar search only included *Salmonella* Typhimurium. Selected strains were from the United States and European countries (including France, UK, England, and Spain). Lastly, HeirCC is a clustering scheme in EnteroBase based on cgMLST where distances between genomes are estimated using the number of shared cgMLST alleles which are linked on a single-linkage clustering criterion [236]. This genomic comparison method allows for analyses of population structures by determining the genomic relatedness, clonal information, and clonal complexity of isolates at different levels of resolution. The cutoff values in stepwise cgMLST allelic distances are identified by calculating a matrix of pairwise allelic distances for all cgST and a matrix for HierCC cluster group number at every level of allelic distance (e.g. HC0, HC1, HC2). There are currently 13 HeirCC levels reported for *Salmonella*, ranging from HC0 to HC2850 [237, 238]. For example, HC0 means there are 0 cgMLST allelic differences and H2850 means there are 2850 allelic differences. *Salmonella* in HC900, HC2000, and HC2850 clusters correspond to the 7-gene MLST (consistent with serovars), super-lineage (serovars grouped together), and subspecies, respectively.

One isolate previously serotyped as *Salmonella* Typhimurium, was classified as *Salmonella* Infantitis by SISTR2 and SeqSero with GHRU. Thus, there are 46 *Salmonella* I 4,[5],12:i:- and 38 *Salmonella* Typhimurium. All *Salmonella* I 4,[5],12:i:- strains (n = 46) were assigned to HC10_2, meaning there were at most 10 cgMLST allelic differences between the isolates. Most of the strains were assigned to HC5_6 (n = 42) further indicating the close relationship with only 5 cgMLST allelic differences. Additionally, three were assigned to HC5_114941 and one HC5_52400. Publicly available *Salmonella* I 4,[5],12:i:- genomes on EnteroBase within these assigned HC5_6, HC5_114941, and HC5_53400 were randomly selected.

655 *Salmonella* I 4,[5],12:i:- strains assigned to HC5_6 were selected, while the *Salmonella* I 4,[5],12:i:- assigned to HC5_114941 and HC5_52400 were found only in our study. Of the 655, 154 had two cgMLST allelic differences with 7 *Salmonella* I 4,[5],12:i:- in our study. *Salmonella* Typhimurium strains with the least allelic differences to the *Salmonella* I 4,[5],12:i:- strains (n = 46) from our study were selected. All selected *Salmonella* Typhimurium strains (n = 208) had a maximum of 200 allelic differences (HC200_2) with all *Salmonella* I 4,[5],12:i:- strains (n = 701), including our strains. Within the 208 selected *Salmonella* Typhimurium strains compared to *Salmonella* I 4,[5],12:i:-, 19 had 100 allelic differences (HC100_2), three had 50 allelic differences (HC50_2), two had 20 allelic differences (HC20_2), and one had 10 allelic differences (HC10_2).

The metadata of the selected *Salmonella* genomes were downloaded from EnteroBase which includes the continent, country, region, lab contact, collection year, source niche, source details, HeirCC, BioProject ID, Project ID, Sample ID, and SRA accession number of each strain. Using the NCBI SRA Toolkit v2.9.2 (https://github.com/ncbi/sra-tools) with the fasterq-dump tool, the SRA accession numbers were used to download the publicly available *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes (FASTQ files) including the strains in our study [239]. The FASTQ files were then processed through pipelines used for analyzing genomic data (as explained below) to have uniform data across all genomes for phylogenetic analyses.

3.4.2. GHRU (Genomic Surveillance of Antimicrobial Resistance) Bioinformatics

The bioinformatics platform used for phylogenetic analyses was from the retrospective 1 project of the National Institute for Health Research (NIHR) Global Health Research Unit (GHRU; Genomic Surveillance of Antimicrobial Resistance) (https://gitlab.com/cgps/ghru/pipelines). The

core analysis of de novo assembly (assembly), mapping based SNP phylogeny (snp_phylogeny), MLST assignment (MLST), and AMR gene detection (ARIBA) were all performed utilizing versioned Nextflow workflows and linked Docker containers (Table 3-2). The pipeline workflow is summarized in GHRU Retrospective 1 Bioinformatics Methods V.4 protocols: https://protocols.io/view/ghru-genomic-surveillance-of-antimicrobial-resista-bpn6mmhe [240].

Briefly, the GHRU pipeline begins by evaluating the quality of all raw reads (FASTQ files) (before and after trimming) using FastQC v0.11.9 [241]. The raw reads were then processed by Trimommatic v0.39 to trim reads and remove the adapters [242], Lighter v1.1 to correct sequencing errors [243], Seqtk v1.3 to downsample to 100x coverage [244], FLASH v1.2.11 for read merging and length adjustment [245], and Mash v2.2 which estimates pairwise mutation distances [246]. Species identification was determined by BactInspector v0.1.3 [247] and the absence of contamination was assessed using and Confidr v0.7.1 [248]. The trimmed FASTQ files were assembled using SPAdes v3.14.0 [249], and the quality of the assembled genomes were evaluated using Quast v5.0.2 [250]. Low quality FASTQ and FASTA files were removed from further analyses. After the GHRU quality assessment, there were a total of 683 *Salmonella* I 4,[5],12:i:- and 242 *Salmonella* Typhimurium genomes, totaling to 925.

AMR genes, point mutations, and MLST profiles were identified using the software ARIBA v2.14 with trimmed FASTQ files [251]. ARIBA v2.14 uses the following databases: ResFinder v4.0 [233], PlasmidFinder v2.1 [252], and PubMLST [253, 254]. This version of the ARIBA software was created by Anthony Underwood [255]. For serovar determination and confirmation, the software SISTR v1.0.2 [256] and SeqSero2 v2 [216] were used with FASTA files. Furthermore, to differentiate between the Spanish, European, and U.S. clones (Table 2-1),

previously described markers for *flj*A, *flj*B, *iro*B, and *fli*C were used via BLAST+ v2.0.9 [13, 257] to determine presence and absence of those particular genes in *Salmonella* strains.

For SNP-based phylogenetic analyses, FASTQ files that passed the quality assessment were mapped to the *Salmonella* Enteritidis str. 18569 (GCA_000335875.2) reference genome using BWA-MEM v0.7.17 [258], and SNP variants were called and filtered using BCFtools v1.9 [259, 260]. The alignment was used to create a maximum likelihood tree using the software IQ-TREE v1.6.12 [261]. All WGS data was integrated, loaded and visualized using Microreact [262] into three separate projects: (1) *Salmonella* I 4,[5],12:i:- (n = 46) and *Salmonella* Typhimurium (n = 38) genomes from our study (https://microreact.org/project/paJQVedBdiNuNZcvDn6yMb), (2) *Salmonella* I 4,[5],12:i:- from our study (n = 46) and publicly available *Salmonella* I 4,[5],12:i:- genomes (n = 637) (https://microreact.org/project/8jV82M8i1feSxCMpYXNNke), and (3) all *Salmonella* I 4,[5],12:i:- (n = 683) as well as *Salmonella* Typhimurium (n = 38) genomes from our study and publicly available *Salmonella* Typhimurium (n = 204) genomes (https://microreact.org/project/prM1DhwDzM175G4vCzcaTR). Subtrees were defined as a sub-section of a phylogenetic tree. Clades were identified when all descendants originated from one node. Clusters were defined as genomes with similar characteristics or resemblance.

Table 3-2 GHRU Retrospective 1 Bioinformatics Methods V.4 - Nextflow workflows

Workflow name	Workflow link	Docker hub Container(s) used	Version at public action
De novo assembly	https://gitlab.com/cgps/ghru/piplines/assembly	bioinformant/ghru-assembly:version OR registry.gitlab.com/cgps/ghru/pipelines/assembly:version	1.5.5
Mapping SNP-based phylogeny	https://gitlab.com/cgps/ghru/piplines/snp_phylogeny	bioinformant/ghru-snp-phylogeny:version OR registry.gitlab.com/cgps/ghru/pipelines/snp_phylogeny:version	1.2.2
AMR determinant detection	https://gitlab.com/cgps/ghru/piplines/amr_prediction	bioinformant/ghru-amr-prediction:version OR registry.gitlab.com/cgps/ghru/pipelines/dsl2/pipelines/amr_prediction	1.0
MLST	https://gitlab.com/cgps/ghru/piplines/mlst	bioinformant/ghru-mlst:version OR registry.gitlab.com/cgps/ghru/pipelines/dsl2/pipelines/mlst:version	1.0

4. RESULTS

4.1. Genotypic serotype and MLST of Salmonella *isolates*

The 125 *Salmonella* isolates were included in this study were traditionally serotyped using the White-Kauffmann-Le Minor scheme by the National Veterinary Services Laboratory, Ames, IA. The following *Salmonella* serovars were confirmed via genotypic serotyping: Agona (n = 7), Alachua (n = 4), Derby (n = 9), Enteritidis (n = 8), Montevideo (n = 3), Senftenberg (n = 10), and Typhimurium (n = 37). The sequence types (STs) were also identified and confirmed for Agona (ST13), Alachua (ST1298), Derby (ST40), Enteritidis (ST11), Montevideo (ST138), Senftenberg (ST14), and Typhimurium (ST19). All but one *Salmonella* I 4,[5],12:i:- isolate (n = 46) was genotypically confirmed as a monophasic variant of Typhimurium. One *Salmonella* I 4,[5],12:i:- isolate was genotypically serotyped as *Salmonella* Typhimurium and grouped with the Typhimurium strains for further analyses. Therefore, the total number of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium was 46 and 38, respectively. The sequence types of all *Salmonella* I 4,[5],12:i:- were ST34 and *Salmonella* Typhimurium were ST19.

4.2. Phenotypic and genotypic antimicrobial resistance

Based on the antimicrobial susceptibility testing, the *Salmonella* I 4,[5],12:i:- isolates had four unique phenotypic resistance profiles, with all isolates resistant to three or more antibiotic classes (Table 4-2). Therefore, all of the *Salmonella* I 4,[5],12:i:- isolates were classified as MDR. One isolate was resistant to streptomycin, sulfonamides, and tetracycline (SSuT), encoded by the *str*A-*str*B, *sul2*, and *tet*(B) genes and also harbored $bla_{\text{TEM-1}}$ and *qnrB*19 (a plasmid-mediated quinolone resistance (PMQR) gene). This isolate was classified as intermediate to enrofloxacin

(MIC = 1 µg/ml) and did not show decreased susceptibility to ciprofloxacin (DSC) (MIC = 0.03 µg/ml). Approximately 98% (45 out of 46) *Salmonella* I 4,[5],12:i:- strains displayed resistance to ampicillin, streptomycin, sulfonamides, and tetracycline (ASSuT) encoded by the *bla*$_{\text{TEM-1}}$, *str*A-*str*B, *sul2*, and *tet*(B) genes (Table 4-3). Of these, only 14 (30.4%) were ASSuT R-type (resistance to only these antibiotics). However, 32 ASSuT resistant *Salmonella* I 4,[5],12:i:- isolates additionally harbored *qnr*B19 with 31 showing decreased susceptibility to ciprofloxacin (DSC); 31 isolates had a MIC of 0.5 µg/ml (67.4% of all *Salmonella* I 4,[5],12:i:- isolates), and one had a MIC of 0.015 µg/ml. The majority of the isolates with a MIC of 0.5 µg/ml for ciprofloxacin were considered intermediate to enrofloxacin with 25 at a MIC of 2 µg/ml (54.4% of all *Salmonella* I 4,[5],12:i:- isolates), while six had a MIC of 1 µg/ml (Table 4-1). Twenty-eight isolates (60.9%) with the ASSuT resistance phenotype and presence of *qnr*B19 had additional resistance to nalidixic acid (ASSuT-Na R-type) (Table 4-2). Moreover, three (6.5%) with the ASSuT resistance phenotype harbored *qnrB*2 instead of *qnrB*19 with additional resistance to trimethoprim-sulfamethoxazole and ceftriaxone (ASSuT-CxSxT R-type). Those three isolates harbored additional resistance genes to streptomycin-spectinomycin (*aadA2*), aminoglycosides (*aph(3')-Ia*), extended spectrum beta-lactams (*bla*$_{\text{SHV-12}}$), sulfonamides (*sul1*), trimethoprim/sulfamethoxazole (*dfrA19*), polymyxins (*mcr*-9), and tetracyclines (*tet*(D)) (Table 4-1). The three isolates that harbored *qnrB*2 showed DSC with a MIC of 0.25 µg/ml for ciprofloxacin rather than a MIC of 0.5 µg/ml that was associated with the *qnrB*19 gene. These isolates were not resistant to nalidixic acid but showed reduced susceptibility with a MIC of 16 µg/ml. Two of these three isolates were considered intermediate to enrofloxacin with a MIC of 1 µg/ml while one was susceptible (MIC of 0.5 µg/ml). Additionally, *Salmonella* I 4,[5],12:i:- isolates with ceftriaxone

resistance were also classified as intermediate to ceftiofur, a beta-lactam antibiotic and third generation cephalosporin (Table 4-1). None of the 46 *Salmonella* I 4,[5],12:i:- isolates contained point mutations in the DNA gyrase (*gyrA* and *gyrB*) or topoisomerase IV genes (*parC* and *parE)*.

Table 4-1 Minimum inhibitory concentration (MIC) distribution (squashtogram) and percentages at CLSI or NARMS breakpoint values of 46 *Salmonella* I 4,[5],12:i:- isolates

CLSI' Antibiotic Class	Antimicrobial Agent	Percentage of isolates"			Distrubution (#) of all isolates with MIC (ug/ml)*														
		S (%)	I (%)	R (%)	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	100.0	0.0	0.0					15	23	6	2								
	Streptomycin	0.0	N/A	100.0													46			
β-Lactam/β-Lactamase I.	Amoxicillin/Clavulanic Acid	100.0	0.0	0.0								1	4	41						
Cephems	Cefoxitin	97.8	2.2	0.0								36	8	1	1					
	Ceftiofur	93.5	6.5	0.0						26	16	1	3							
	Ceftriaxone	93.5	0.0	6.5					43				2	1						
Folate Pathway Inhibitors	Sulfisoxazole	100.0	N/A	0.0															46	
	Trimethoprim/Sulfamethoxazole	93.5	N/A	6.5				39	4				3							
Macrolides	Azithromycin	100.0	N/A	0.0								10	31	5						
Penicillins	Ampicillin	2.2	0.0	97.8								1				45				
Phenicols	Chloramphenicol	95.7	4.3	0.0									3	41	2					
Quniolones	Ciprofloxacin	100.0	0.0	0.0	10	2			3	31										
	Enrofloxacin	26.1	73.9	0.0				11		1	9	25								
	Nalidixic Acid	39.1	N/A	60.9									11	4	3	28				
Tetracyclines	Tetracycline	0.0	0.0	100.0												46				

Squashtogram showing the distribution of MICs for 14 antimicrobials tested using the CMV3AGNF plate plus Enrofloxacin using BOPO6F plate
One vertical red bar (|) susceptible (S), growth beyond the double vertical red bars (||) resistant (R), in between is intermediate (I)
'CLSI: Clinical and Laboratory Standards Institute
" Percentage of isolates that were susceptible (S), intermediate (I), and resistant (R)
* Unshaded areas indicate dilution range of Sensititre® plates used to test isolates. Single vertical bars indicate breakpoints for susceptibility, while double vertical bars indicate breakpoints for resistance. CLSI breakpoints were used when available or else NARMS consensus breakpoints

Table 4-2. *Salmonella* I 4,[5],12:i:- phenotypic and genotypic resistance profiles

Phenotypic resistance profiles No. (%)		Genotypic resistance profiles No. (%)	
SSuT	1 (2.2)	*bla*$_{TEM-1}$, *qnrB*19, *strA-strB*, *sul2*, *tet*(B)[†]	1 (2.2)
ASSuT	14 (30.4)	*aadA1*, *bla*$_{TEM-1}$, *strA-strB*, *sul1*, *sul2*, *tet*(B)	1 (2.2)
		aph(3')-Ia, *bla*$_{TEM-1}$, *strA-strB*, *sul2*, *tet*(B)	1 (2.2)
		bla$_{TEM-1}$, *qnrB*19, *strA-strB*, *sul2*, *tet*(B)[†]	4 (8.7)
		bla$_{TEM-1}$, *strA-strB*, *sul2*, *tet*(B)	8 (17.4)
ASSuT-Na	28 (60.9)	*bla*$_{TEM-1}$, *qnrB*19, *strA-strB*, *sul2*, *tet*(B)[†]	28 (60.9)
ASSuT-CxSxT	3 (6.5)	*aadA2*, *aph(3')-Ia*, *bla*$_{TEM-1}$, *bla*$_{SHV-12}$, *dfrA19*, *mcr*-9, *qnrB*2, *strA-strB*, *sul1*, *sul2*, *tet*(B), *tet*(D)	3 (6.5)

Antibiotic abbreviations: ampicillin (A), ceftriaxone (Cx), streptomycin (S), sulfonamides (Su), tetracycline (T), trimethoprim/sulfamethoxazole (SxT), nalidixic acid (Na)

[†]Total of 33 (69.6%) *Salmonella* I 4,[5],12:i:- presented this genotype with three different phenotypes

In contrast to *Salmonella* I 4,[5],12:i:-, the *Salmonella* Typhimurium isolates had 10 different phenotypic resistance profiles. Of the 37 *Salmonella* Typhimurium isolates selected for this study, nine were pan-susceptible (23.7%), 29 isolates were resistant to at least one antibiotic (76.3%), and 27 of the 29 (71.1%) resistant isolates were MDR (Table 4-4). One *Salmonella* Typhimurium isolate was resistant to only streptomycin but did not have any resistance genes based on the search engines used in this study. One other isolate harbored *aadA1*, *strA*, *strB*, *sul1*, *tet*(A) but only showed resistance to streptomycin and tetracycline (Table 4-4). Eleven isolates (28.9%) showed resistance to streptomycin, sulfonamides, and tetracycline (SSuT R-type), encoded by *aadA*1, *strA-strB*, *sul1,* and *tet*(A) (Table 4-6). Fifteen *Salmonella* Typhimurium isolates (39.5%) showed phenotypic resistance to ampicillin, streptomycin, sulfonamides, and

tetracyclines (ASSuT). Resistance to ampicillin was encoded by the beta-lactamase genes *bla*$_{\text{CARB-2}}$, *bla*$_{\text{CMY-2}}$, or *bla*$_{\text{TEM-1}}$. Aminoglycoside resistance was encoded by *aadA1* or *aadA2*, while sulfonamide resistance was encoded by *sul1, sul2,* or both. Tetracycline resistance was encoded by either *tet*(A), *tet*(B), *tet*(G), or both *tet*(A) and *tet*(B) in the case of one isolate. Eight of the 15 isolates with ASSuT resistance showed additional resistance to chloramphenicol encoded by *floR* (ACSSuT R-type), with two isolates showing additional resistance to either amoxicillin (ASSuT-Au R-type) or cefoxitin (ASSuT-Cn R-type) (Table 4-4). There are four *Salmonella* Typhimurium isolates with the ASSuT resistance phenotypic that harbored additional resistance genes to streptomycin-spectinomycin (*aadA2*), aminoglycosides (*aph(3')-Ia* and *aac(6')-IIc*), ampicillin (*bla*$_{\text{TEM-1}}$), extended spectrum beta-lactams (*bla*$_{\text{SHV-12}}$), fluoroquinolones (*qnrB*2), sulfonamides (*sul2*), trimethoprim/sulfamethoxazole (*dfrA*19), and polymyxins (*mcr*-9). The tetracycline resistance gene present in these four isolates was *tet*(D). There were two different phenotypic profiles within these four isolates including resistance to ceftriaxone and trimethoprim/sulfamethoxazole (ASSuT-CxSxT R-type) and another with additional resistance to ceftiofur (ASSuT-CrCxSxT R-type). Of the four *Salmonella* Typhimurium isolates with the *bla*$_{\text{SHV-12}}$ gene, all showed phenotypic resistance to ceftriaxone and were also classified as resistant (n = 3) or intermediate to ceftiofur (n = 1) (Table 4-3). Additionally, these three *Salmonella* Typhimurium isolates showed DSC with a MIC of 0.25 µg/ml to ciprofloxacin and nine were considered intermediate to enrofloxacin with a MIC of 1 µg/ml (Table 4-3). Although not resistant to nalidixic acid, these isolates showed reduced susceptibility with a MIC of 8 µg/ml (n = 2) or 16 µg/ml (n = 2). None of the 38 *Salmonella* Typhimurium isolates contained point mutations in the DNA gyrase (*gyrA* and *gyrB*) or topoisomerase IV genes (*parC* and *parE*).

Table 4-3 Minimum inhibitory concentration (MIC) distribution (squashtogram) and percentages at CLSI or NARMS breakpoint values of *Salmonella* Typhimurium isolates

CLSI' Antibiotic Class	Antimicrobial Agent	Percentage of isolates"			Distrubution (#) of all isolates with MIC (ug/ml)*														
		S (%)	I (%)	R (%)	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512
Aminoglycosides	Gentamicin	100.0	0.0	0.0					1	20	17									
	Streptomycin	26.3	N/A	73.7										2	8	1	27			
β-Lactam/β-Lactamase I.	Amoxicillin/Clavulanic Acid	68.4	26.3	5.3							23			3	10	2				
Cephems	Cefoxitin	94.7	0.0	5.3								26	9	1		2				
	Ceftiofur	89.5	2.6	7.9							33	1	1	3						
	Ceftriaxone	89.5	0.0	10.5					34				2	1	1					
Folate Pathway Inhibitors	Sulfisoxazole	100.0	N/A	0.0												10	2		26	
	Trimethoprim/Sulfamethoxazole	92.1	N/A	7.9				13	20	2			3							
Macrolides	Azithromycin	100.0	N/A	0.0								2	29	7						
Penicillins	Ampicillin	60.5	0.0	39.5							7	16				15				
Phenicols	Chloramphenicol	55.3	15.8	28.9								20		1	6	11				
Quniolones	Ciprofloxacin	100.0	0.0	0.0	30	5		3												
	Enrofloxacin	84.2	15.8	0.0				24	1	7	6									
	Nalidixic Acid	100.0	N/A	0.0								3	31	3	1					
Tetracyclines	Tetracycline	26.3	0.0	73.7									10			28				

Squashtogram showing the distribution of MICs for 14 antimicrobials tested using the CMV3AGNF plate plus Enrofloxacin using BOPO6F plate
One vertical red bar (|) susceptible (S), growth beyond the double vertical red bars (||) resistant (R), in between is intermediate (I)
'CLSI: Clinical and Laboratory Standards Institute
"Percentage of isolates that were susceptible (S), intermediate (I), and resistant (R)
*Unshaded areas indicate dilution range of Sensititre® plates used to test isolates. Single vertical bars indicate breakpoints for susceptibility, while double vertical bars indicate breakpoints for resistance. CLSI breakpoints were used when available or else NARMS consensus breakpoints

Table 4-4 *Salmonella* Typhimurium phenotypic and genotypic resistance profiles

Phenotypic resistance profiles No. (%)		**Genotypic resistance profiles No. (%)**	
Pan-susceptible	9 (24.3)	No resistance genes	9 (24.3)
S	1 (2.7)	No resistance genes	1 (2.7)
ST	1 (2.7)	*aadA1*, *strA-strB*, *sul1*, *tet*(A) [‡]	1 (2.7)
SSuT	11 (29.7)	*aadA1*, *strA-strB*, *sul1*, *tet*(A) [‡]	10 (27)
		aadA1, *strA-strB*, *sul1*, *tet*(A), *tet*(B)	1 (2.7)
ASSuT-CxSxT	2 (5.4)	*aadA2*, *aph(3')-Ia*, *aac(6')-IIc*, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-12}}$, *dfrA19, mcr*-9, *qnrB*2, *ereA*, *strA-strB*, *sul1*, *sul2*, *tet*(D)[†]	2 (5.4)
ASSuT-CrCxSxT	2 (5.4)	*aadA2*, *aph(3')-Ia*, *aac(6')-IIc*, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-12}}$, *dfrA19, mcr*-9, *qnrB*2, *ereA*, *strA-strB*, *sul1*, *sul2*, *tet*(D)[†]	2 (5.4)
ASSuT-AuCnCrCx	1 (2.7)	*aadA1*, *bla*$_{\text{CMY-2}}$, *strA-strB*, *sul1*, *tet*(A)	1 (2.7)
ACSSuT	9 (24.3)	*aadA2*, *bla*$_{\text{CARB-2}}$, *floR, sul1*, *tet*(G)[§]	8 (21.1)
		aadA2, *floR, strA*, *sul1*, *tet*(G)	1 (2.7)
ACSSuT-Au	1 (2.7)	*aadA2*, *bla*$_{\text{CARB-2}}$, *floR, sul1*, *tet*(G)[§]	1 (2.7)
ACSSuT-Cn	1 (2.7)	*aadA2*, *bla*$_{\text{CARB-2}}$, *floR, sul1*, *tet*(G)[§]	1 (2.7)

Antibiotic abbreviations: ampicillin (A), amoxicillin/clavulanic acid (Au), cefoxitin (Cn), ceftriaxone (Cx), ceftiofur (Cr), chloramphenicol (C), streptomycin (S), sulfonamides (Su), tetracycline (T), trimethoprim/sulfamethoxazole (SxT) streptomycin (S), sulfonamides (Su), tetracycline (T)

[†]Total of 4 (10.8%) *Salmonella* Typhimurium presented this genotype with two different phenotypes

[‡]Total of 11 (29.7%) *Salmonella* Typhimurium presented this genotype with two different phenotypes

[§]Total of 10 (27%) *Salmonella* Typhimurium presented this genotype with three different phenotypes

In summary, all the *Salmonella* I 4,[5],12:i:- isolates were MDR with approximately 98% showing phenotypic resistance to ampicillin, streptomycin, sulfonamides, and tetracycline with the corresponding resistance genes as seen in Figure 4-1 and Figure 4-2. More than half (60.9%) were also resistant to nalidixic acid, while a small percentage (6.5%) were resistant to trimethoprim-sulfamethoxazole. In comparison, *Salmonella* Typhimurium had more diverse phenotypic profiles with both pan-susceptible (24.3%) and MDR (72.9%) strains. *Salmonella* Typhimurium isolates showed resistance to additional antibiotics compared to *Salmonella* I 4,[5],12:i:- such as amoxicillin/clavulanic acid, cefoxitin, ceftiofur, and chloramphenicol (Figure 4-1). Furthermore, while both *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium harbored multiple AMR genes, there were distinct genes present within each serotype (Figure 4-2). All *Salmonella* I 4,[5],12:i:- isolates harbored the $bla_{\text{TEM-1}}$, *strA-strB*, *sul2*, and *tet*(B) genes encoding phenotypic resistance to ampicillin, streptomycin, sulfonamides, and tetracycline. In contrast, AMR genes varied across *Salmonella* Typhimurium isolates as seen in Figure 4-2. As opposed to *sul1* (6.5%) and *sul2* (100%) in *Salmonella* I 4,[5],12:i:- isolates, 73.7% of *Salmonella* Typhimurium isolates harbored the *sul1* gene, while only 10.5% contained the *sul2* gene. Streptomycin-spectinomycin resistance genes also differed between the two serotypes. *Salmonella* I 4,[5],12:i:- harbored *strA-strB* (100%), *aadA2* (8.7%), and *aadA1* (2.2%), while *Salmonella* Typhimurium harbored *strA* (47.4%), *strB* (44.7%), *aadA1* (34.2%), and/or *aadA2* (36.8%). Furthermore, all *Salmonella* I 4,[5],12:i:- isolates showed presence of the *tet*(B) resistance gene with three also

containing *tet*(D); whereas, *Salmonella* Typhimurium harbored four different tetracycline genes, with most containing *tet*(A) (31.6%) followed by *tet*(G) (28.9%), *tet*(D) (10.5%), and *tet*(B) (2.6%). The majority of *Salmonella* I 4,[5],12:i:- isolates also contained the PMQR genes, *qnrB*19 (69.6%) and *qnrB*2 (6.5%). In contrast to *Salmonella* I 4,[5],12:i:-, a few *Salmonella* Typhimurium isolates harbored only the *qnrB*2 (10.5%) gene. There were *Salmonella* I 4,[5],12:i:- (three isolates) and *Salmonella* Typhimurium (four isolates) isolates harboring more than 10 AMR genes. Common AMR genes among both serotypes were: *aadA2*, *aph(3')-Ia*, $bla_{\text{TEM-1}}$, $bla_{\text{SHV-12}}$, *dfrA19*, *mcr*-9, *qnrB*2, *strA*, *strB*, *sul1*, *sul2*, and *tet*(D). Only *Salmonella* Typhimurium contained the following genes: *aac(6')-IIc*, $bla_{\text{CARB-2}}$, $bla_{\text{CMY-2}}$, *ere*(A), and *floR.* All *Salmonella* I 4,[5],12:i:- had the *tet*(B) gene, while only one *Salmonella* Typhimurium had the *tet*(B) gene.

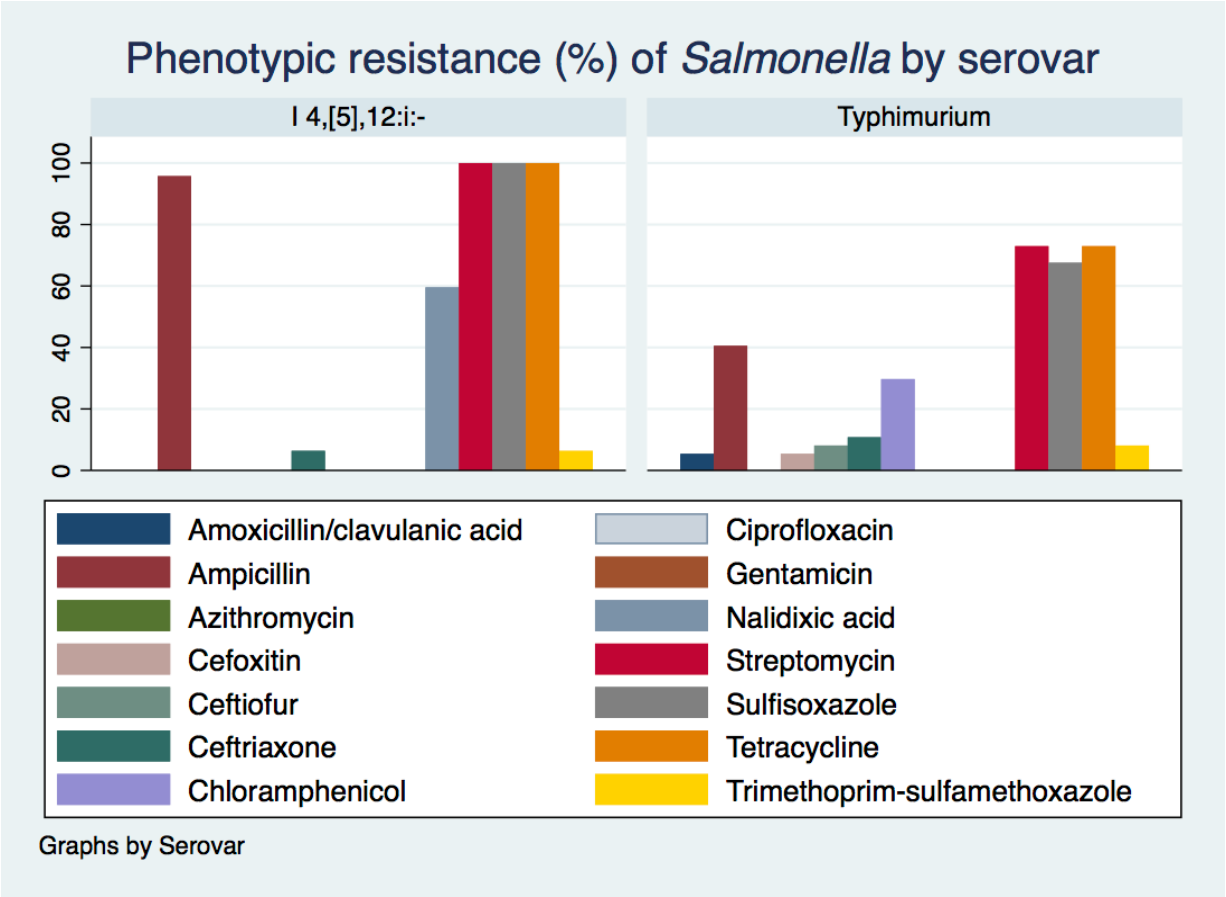


Figure 4-1 Comparison of phenotypic resistance between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates

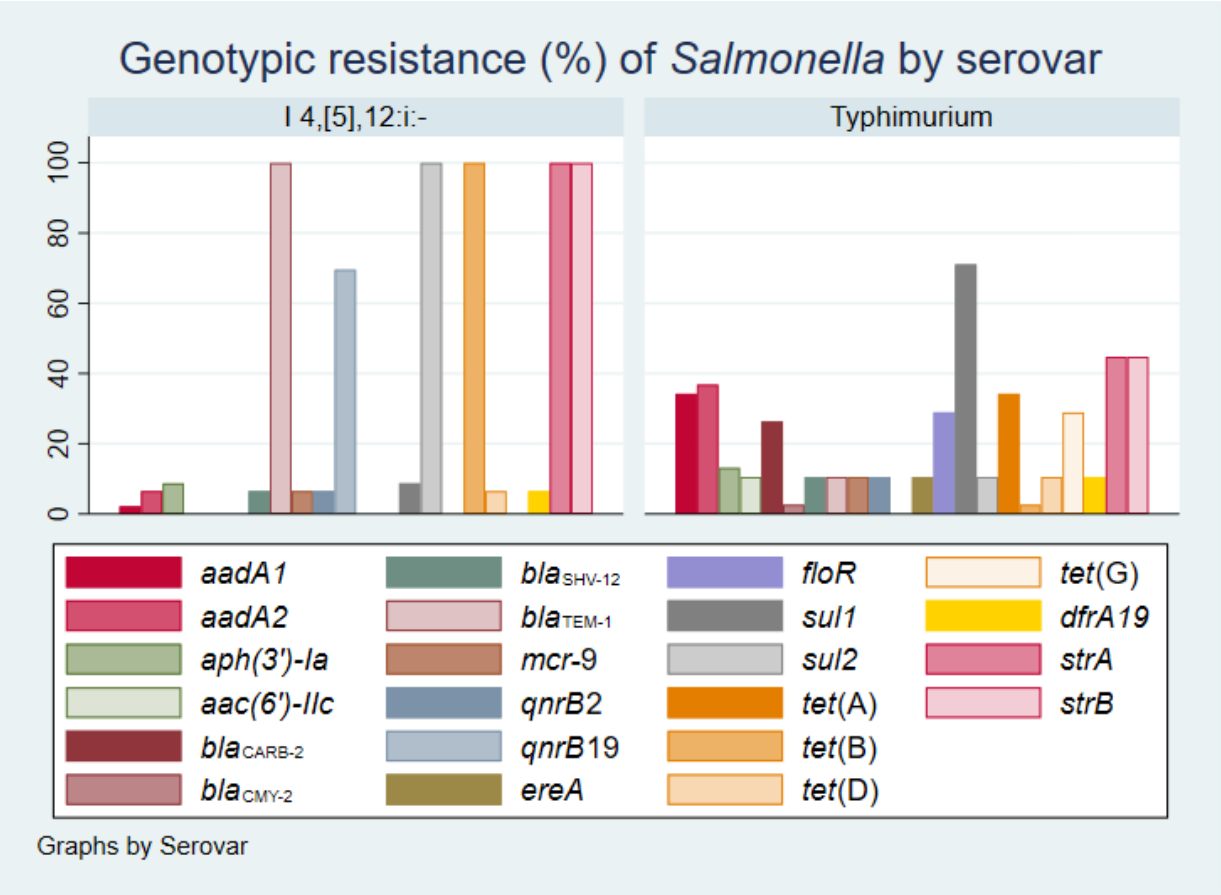


Figure 4-2 Comparison of genotypic resistance between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates

The other monophasic *Salmonella* serovars included in this study varied in phenotypic and genotypic resistance. All *Salmonella* Agona isolates were MDR with six of the seven isolates showing phenotypic resistance to streptomycin, sulfonamides and tetracyclines (SSuT R-type) encoded by the *aadA7*, *sul1*, and *tet*(A) resistance genes and harbored the fosfomycin resistance gene, *fosA7*. Only one *Salmonella* Agona isolate showed resistance to ACSSuT encoded by the corresponding *bla*$_{\text{TEM-1}}$, *floR*, *strA* and *strB*, *sul1* and *sul2*, and *tet*(A) and *tet*(B) genes and had additional resistance to amoxicillin, cefoxitin, ceftriaxone, ceftiofur, and gentamicin (ACSSuT-AuCnCrCxG R-type). This one *S*. Agona isolate also harbored additional resistance genes to aminoglycosides (*aac(3)-IV*, *aph(3')-Ia*, and *aph(4)-Ia*), cephalosporins such as cefoxitin, ceftriaxone and ceftiofur (*bla*$_{\text{CMY-2}}$), and lastly fosfomycin (*fosA7*). All *Salmonella* Agona isolates contained a *parC* point mutation and the corresponding MICs to nalidixic acid were 2 µg/ml (n = 2), 4 µg/ml (n = 4), and 8 µg/ml (n = 1). In addition, all were susceptible to ciprofloxacin with MICs of 0.015 µg/ml (n = 6) and 0.03 µg/ml (n = 1).

All four *Salmonella* Alachua isolates were MDR showing resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, amoxicillin/clavulanic acid, cefoxitin, ceftiofur, and ceftriaxone (ACSSuT-AuCnCrCxGSxT R-type) encoded by the following genes: *aac(3)-IId*, *aadA2*, *aph(3')-Ia, bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$, *dfrA12, floR*, *strA-strB*, *sul1* and *sul2*, and *tet*(A) and *tet*(B). Additionally, all contained the fosfomycin

resistance gene, *fosA7*. *Salmonella* Alachua isolates resistant to ceftriaxone had MICs of 64 µg/ml (n = 3) and 32 µg/ml (n = 1). All *Salmonella* Alachua isolates contained a *parC* point mutation and were susceptible to ciprofloxacin with MICs of 0.015 µg/ml (n = 4) and nalidixic acid with MICs of 4 µg/ml (n = 3) and 16 µg/ml (n = 1).

There were four different phenotypic resistance profiles for *Salmonella* Derby isolates (n = 9). Five isolates were MDR showing resistance to SSuT (*aadA2* or *aadA7, sul1* and *tet*(A)), with one isolate showing additional resistance to nalidixic acid (SSuT-Na R-type). The *fosA7* gene was also present in these isolates. Three other isolates only contained the *fosA7* gene with two being pan-susceptible and one resistant to streptomycin. The isolate with phenotypic resistance to nalidixic acid harbored the PMQR gene *qnrB*19, and showed DSC with a MIC of 0.5 µg/ml. One isolate was resistant to only tetracycline and harbored the *tet*(B) gene. Lastly, two *Salmonella* Derby isolates were pan-susceptible to the 14 antimicrobials tested with the NARMS CMV3AGNF plate. All *Salmonella* Derby isolates contained a *parC* point mutation and were susceptible to ciprofloxacin with MICs of 0.015 µg/ml (n = 7), 0.03 µg/ml (n = 1), and 0.05 µg/ml (*qnrB19,* n = 1). The eight *Salmonella* Derby isolates that were susceptible to nalidixic acid had MICs of 2 µg/ml (n = 3), 4 µg/ml (n = 4), and 8 µg/ml (n = 1).

All *Salmonella* Enteritidis (n = 9) and *Salmonella* Montevideo (n = 3) isolates were pan-susceptible and contained no resistance genes. However, all *Salmonella* Montevideo

contained a *parC* point mutation. The MICs for ciprofloxacin and nalidixic acid were 0.015 µg/ml and 4 µg/ml, respectively for all three *S*. Montevideo isolates.

Salmonella Senftenberg isolates (n = 10) had seven phenotypic resistance profiles. Six isolates were phenotypically resistant to ACSSuT encoded by the *bla*$_{\text{TEM-1}}$, *catA2*, *strA-strB*, *sul1* and *sul2*, and *tet*(D) resistance genes. These *Salmonella* Senftenberg isolates also contained additional resistance genes to aminoglycosides (*aac(6')-IIc*, *aadA2*, and *aph(3')-Ia*), extended spectrum beta-lactams (*bla*$_{\text{SHV-12}}$), colistin (*mcr*-9), macrolides (*ere*A), fluoroquinolones (*qnrB*2 and *aac(6')-Ib-cr*), and trimethoprim/sulfamethoxazole (*dfrA19*). Additionally, two isolates harbored the *tet*(A) gene, and one of these had the *qnrB*19 gene. Isolates containing the PMQR genes and *aac(6')-Ib-cr* showed DSC (MICs of 0.5 µg/ml) and two were resistant to nalidixic acid, while three showed reduced susceptibility with MICs of 16 µg/ml (ASSuT-CrCxGNaSxT R-type). Only one of these six isolates showed additional resistance to azithromycin and cefoxitin (ACSSuT-AzCnCrCxGSxT R-type). Furthermore, one *Salmonella* Senftenberg isolate was phenotypically resistant to ciprofloxacin, streptomycin, sulfonamides, and tetracycline (SSuT) and harbored the corresponding resistance genes *strA* and *strB*, *sul2*, and *tet*(A)); additionally, the isolate had resistance to nalidixic acid (*qnrB*19) and DSC with a MIC of 1 µg/ml. One *Salmonella* Senftenberg contained no resistance genes, however, was phenotypically resistant to chloramphenicol. One other was pan-susceptible

but contained the *aph(3')-Ia* gene. All *Salmonella* Senftenberg isolates contained a *parC* point mutation.

Table 4-5 Number and percentage of *Salmonella* isolates with selected phenotypic resistance patterns, by serotype

Serotype		At least SSuT'	At least ASSuT"	At least ACSSuT[†]	At least ACSSuT-AuCx[‡]	At least DSC[§]	At least Cx	At least DSC[§] and Cx
	n	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Agona	7	7 (100)	0 (0)	1 (14.3)	1 (14.3)	0 (0)	1 (14.3)	0 (0)
Alachua	4	0 (0)	0 (0)	4 (100)	4 (100)	0 (0)	0 (0)	0 (0)
Derby	9	5 (55. 6)	0 (0)	0 (0)	0 (0)	1 (11.1)	0 (0)	0 (0)
Enteritidis	8	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
I 4,[5],12:i:-	46	46 (100)	45 (97.8)	0 (0)	0 (0)	34 (73.9)	3 (6.5)	3 (6.5)
Montevideo	3	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Senftenberg	10	8 (80)	2 (20)	7 (70)	7 (70)	8 (80)	7 (70)	7 (70)
Typhimurium	38	25 (65.8)	17 (44.7)	8 (21.1)	12 (31.6)	3 (7.9)	5 (13.2)	3 (7.9)

SSuT' phenotype: streptomycin (S), sulfonamides (Su), tetracycline (T)
ASSuT" phenotype: ampicillin (A), streptomycin (S), sulfonamides (Su), tetracycline (T),
ACSSuT[†] phenotype: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), tetracycline (T),
ACSSuT-AuCx[‡] phenotype: ampicillin (A), chloramphenicol (C), streptomycin (S), sulfonamides (Su), tetracycline (T), amoxicillin/clavulanic acid (Au), ceftriaxone (Cx)
DSC[§] phenotype: decreased susceptibility to ciprofloxacin (DSC)

4.3. Fitness costs of antimicrobial resistance genes

The fitness cost of the antimicrobial resistance genes was assessed using the Bioscreen C^{TM} Automated Microbiology Growth Curve Analysis System (Bioscreen C). The baseline curve (blue) in all growth curve graphs represents all the isolates of a particular *Salmonella* serovar grown in the Muller-Hintion Broth medium without any antibiotic over a 48-hour period. The remaining curves represent the *Salmonella* isolates of one serovar, which harbor the respective AMR gene(s), encoding resistance to the antibiotic in the medium at different concentrations (µg/ml). For example, fitted growth curves for *Salmonella* isolates with PMRQ genes (*qnrB2, qnrB19* and *aac-Ib-cr*) and/or point mutation (*parC*) for enrofloxacin (0.25 to 4 µg/ml), beta-lactamase resistance genes ($bla_{\text{SHV-12}}$) for ceftiofur (2 to 8 µg/ml), and tetracycline resistance genes (*tet*(A), *tet*(B), *tet*(D), and *tet*(G)) for tetracycline (4 to16 µg/ml). The fitness cost of an AMR gene was determined by comparing the relative growth rates (henceforth, growth rate) of *Salmonella* isolates at baseline with the growth of *Salmonella* isolates with the respective AMR gene with no antibiotic. Significant differences were determined when the 95% CI of the following variables did not overlap: b_1 (stationary phase), b_2 (exponential phase or slope), and b_3 (lag phase). The growth rate is defined as the change in number of cells per minute, which is estimated by the change in OD per minute.

*4.3.1. Enrofloxacin: PMRQ genes (*qnrB, aac-Ib-cr*), and point mutation (*parC*)*

4.3.1.1. Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

The growth rate of *Salmonella* I 4,[5],12:i:- isolates with the PMQR resistance gene *qnrB*19 (n = 33) was compared to the baseline, and as seen in Figure 4-3, there is no fitness cost to harboring *qnrB*19 for *Salmonella* I 4,[5],12:i:- isolates. At concentrations of 0.25 µg/ml and 0.50 µg/ml of enrofloxacin, bacterial growth was slightly affected at the exponential and stationary phases when compared to the baseline ($p \leq 0.05$) (Figure 4-3). However, there were no significant differences at the lag phase between the baseline growth rates and the growth rates at 0.25 µg/ml and 0.5 µg/ml of enrofloxacin. As the enrofloxacin concentration increased to 1 and 2 µg/ml, the growth rates decreased significantly at the lag, exponential and stationary phase ($p \leq 0.05$) and eventually there was no growth at 4 µg/ml. At lower concentrations of enrofloxacin, the fitness of *Salmonella* I 4,[5],12:i:- isolates with *qnrB*19 was greater and significantly different ($p \leq 0.05$) than at higher concentrations.

Furthermore, the 2 µg/ml enrofloxacin growth curve is unique. Prior to the 1000-minute, growth was nonexistent and like the growth at 4 µg/ml. However, after 1000 minutes, the growth rate increased exponentially and then reached the stationary phase. Although the length of the lag phase (at 2 µg/ml) is longer than at the other concentrations, the eventual exponential growth indicates *Salmonella* I 4,[5],12:i:- with *qnrB*19 was able to eventually grow in the 2 µg/ml enrofloxacin environment. Overall, these fitted growth curves demonstrate that there was no bacterial fitness cost associated with harboring the *qnrB*19 gene at low concentrations of enrofloxacin, as their growth rates did not differ

significantly when compared to growth in the absence of enrofloxacin. However, even strains harboring the *qnrB19* gene had inhibited growth at higher concentrations. The majority of *Salmonella* I 4,[5],12:i:- with *qnrB*19 had a MIC of 2 µg/ml (n = 25) for enrofloxacin, while seven had a MIC of 1 µg/ml and one had a MIC of 0.12 µg/ml.

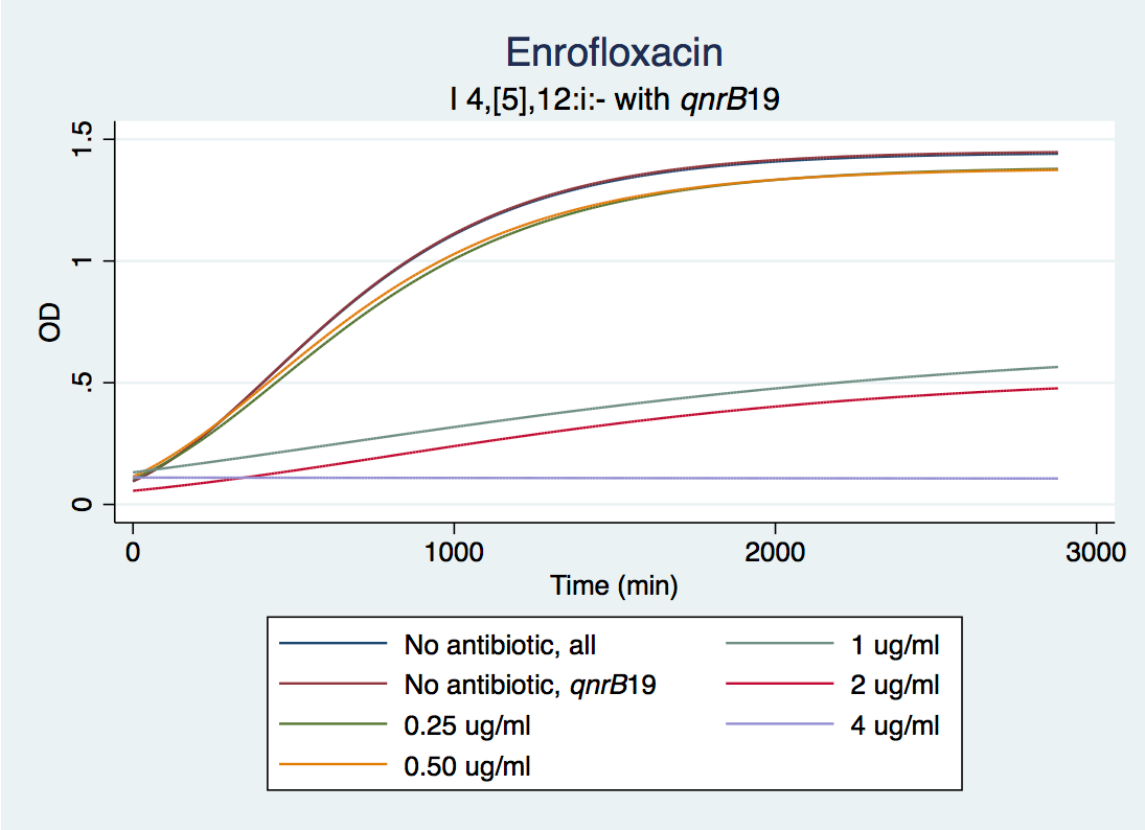


Figure 4-3 3-Parameter Gompertz fitted growth curves of *Salmonella* I 4,[5],12:i:- with the *qnrB*19 gene at different enrofloxacin concentrations

The growth of *Salmonella* I 4,[5],12:i:- isolates with the *qnrB2* gene (n = 3) with no antibiotic compared to the baseline, shows that like the *qnrB19* gene, there is no fitness

cost to harboring the *qnrB*2 gene (Figure 4-4). In comparison to the baseline, all phases of the curve at 0.25 and 0.50 µg/ml were significantly different ($p \leq 0.05$), and the growth rate decreased in comparison to the baseline. There were significant differences in growth for the lag, exponential, and stationary phases comparing growth at 0.25 µg/ml and 0.50 µg/ml of enrofloxacin ($p \leq 0.05$) (Figure 4-4). The growth rate reduced significantly at the lag and stationary phases at 0.50 µg/ml, yet increased at the exponential phase when compared to growth rates at 0.25 µg/ml. Finally, at the higher concentrations of 1, 2, and 4 µg/ml the bacterial strains were unable to grow. Only three *Salmonella* I 4,[5],12:i:- had the *qnrB2* gene and had MICs of 0.5 µg/ml (n = 1) and 1 µg/ml (n = 2).

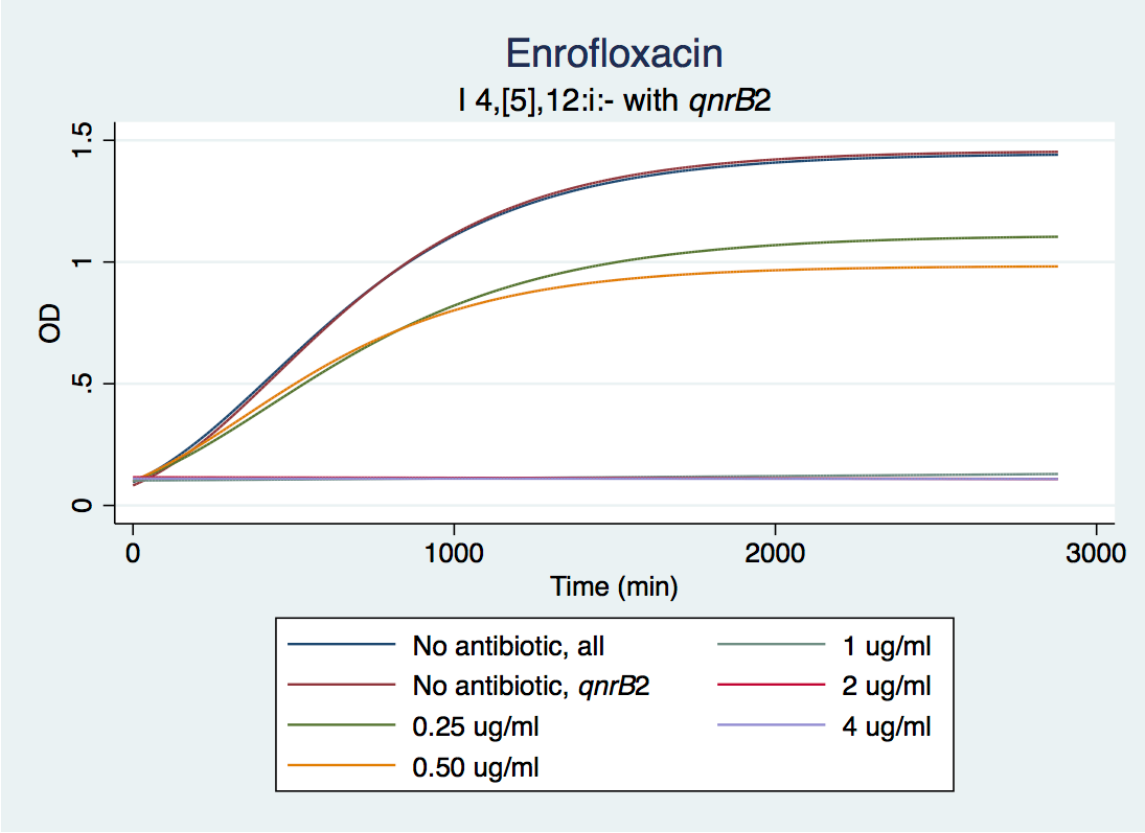


Figure 4-4 3-Parameter Gompertz fitted growth curves of *Salmonella* I 4,[5],12:i:- with the *qnrB*2 gene at different enrofloxacin concentrations

It is important to note that there were significant differences ($p \leq 0.05$) in the growth rates between *Salmonella* I 4,[5],12:i:- isolates harboring *qnrB*2 and those harboring *qnrB*19 at all phases and concentrations of enrofloxacin as seen in Figure 4-5. Growth rates at 0.25 and 0.50 µg/ml for *Salmonella* I 4,[5],12:i:- with *qnrB*2 were significantly less than *Salmonella* I 4,[5],12:i:- with *qnrB*19. *Salmonella* I 4,[5],12:i:- with *qnrB*19 also grew at higher concentrations (1 and 2 µg/ml) while *Salmonella* I 4,[5],12:i:-

with *qnrB*2 stopped growing at 1 µg/ml. Therefore, *Salmonella* I 4,[5],12:i:- harboring *qnrB*19 had increased fitness compared to those harboring *qnrB*2 when grown in enrofloxacin.

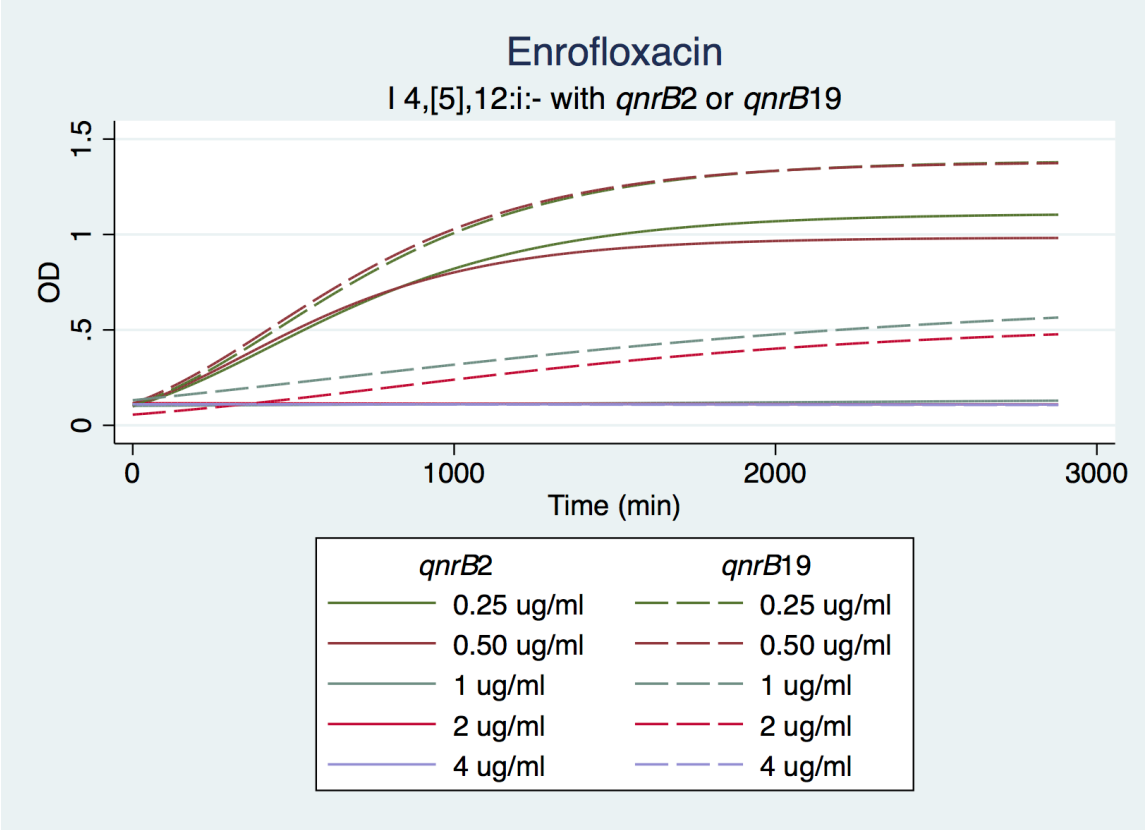


Figure 4-5 Fitness comparison of *Salmonella* I 4,[5],12:i:- with *qnrB*2 and Salmonella I 4,[5],12:i:- with *qnrB*19 at different enrofloxacin concentrations

Figure 4-6 shows how enrofloxacin affects growth of *Salmonella* Typhimurium with *qnrB*2 at different concentrations of enrofloxacin. As opposed to *Salmonella* I 4,[5],12:i:- with *qnrB*2 and even *qnrB*19, a small growth rate reduction is observed at all

phases ($p \leq 0.05$) in no antibiotic for *Salmonella* Typhimurium with *qnrB*2 when compared to the baseline curve (Figure 4-6). Thus, *qnrB2* had a minor fitness cost for *Salmonella* Typhimurium. Interestingly, at 0.25 ug/ml the growth rate is significantly greater than with no antibiotic present indicating *Salmonella* Typhimurium with *qnrB*2 had increased fitness at 0.25 ug/ml. However, as the concentrations of enrofloxacin increased, the growth rates decreased significantly ($p \leq 0.05$) at all phases. The growth rates were significantly different ($p \leq 0.05$) between 0.25 and 0.5 µg/ml for *Salmonella* Typhimurium. A significant decrease in the growth is observed at 0.5 µg/ml and further at 2 µg/ml ($p \leq 0.5$), with 2 µg/ml of enrofloxacin completely inhibiting growth. The growth curve at 1 µg/ml was similar to the growth curve of *Salmonella* I 4,[5],12:i:- with *qnrB*19 at 2 µg/ml in that growth was nonexistent and increased exponentially over time reaching the stationary phase. In this case, the eventual exponential growth indicates *Salmonella* Typhimurium with *qnrB*2 was able to adapt and grow at 1 µg/ml enrofloxacin environment. Four *Salmonella* Typhimurium strains were found to harbor the *qnrB2* gene and all had MICs of 1 µg/ml.

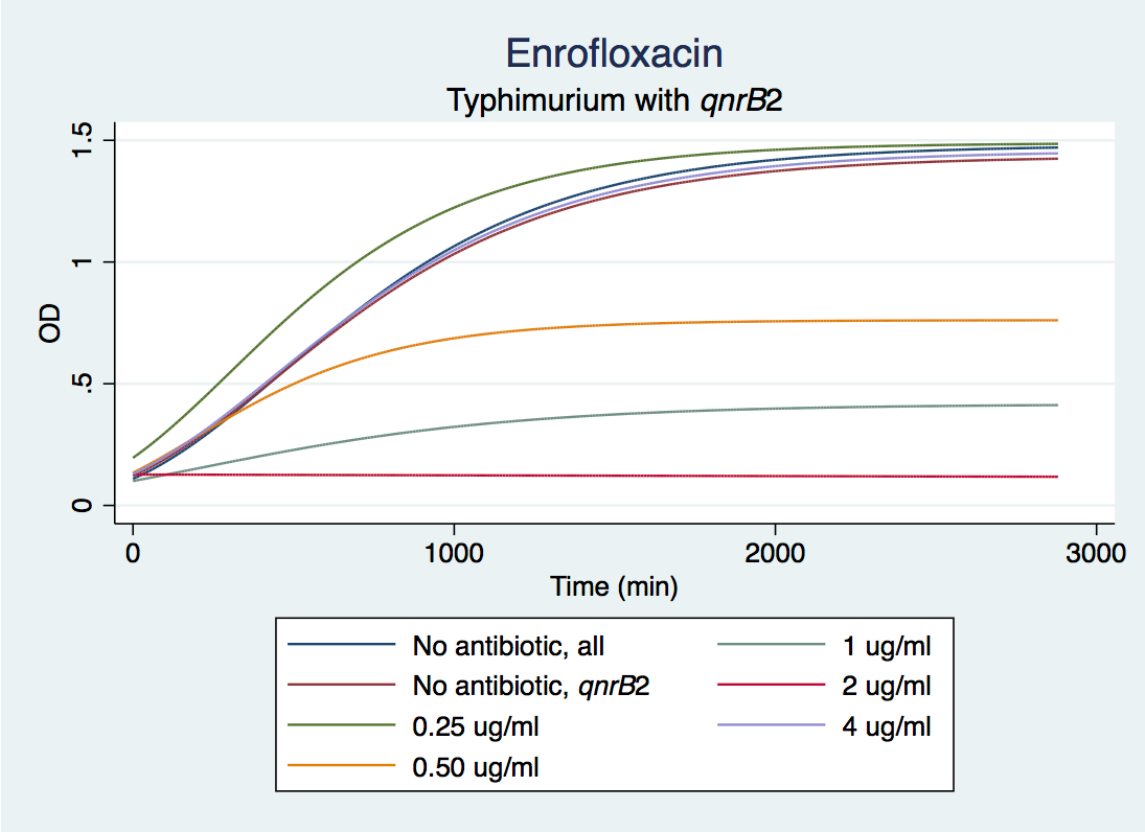


Figure 4-6 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the qnrB2 gene at different enrofloxacin concentrations

Although *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium harbored the *qnrB*2 gene, the fitness for each serovar differed across the concentrations of enrofloxacin (Figure 4-7). The *qnrB*2 gene increased the fitness of *Salmonella* Typhimurium at the smallest concentration of enrofloxacin (0.25 µg/ml) whereas, the growth rate for *Salmonella* I 4,[5],12:i:- decreased. However, the growth rate of *Salmonella* I 4,[5],12:i:- at 0.5 µg/ml remains similar to 0.25 µg/ml while there was a substantial decrease at 0.5

µg/ml for *Salmonella* Typhimurium. Interestingly, *Salmonella* I 4,[5],12:i:- growth was inhibited at 1 µg/ml while *Salmonella* Typhimurium continued to grow and was inhibited at 2 µg/ml. The presence of *qnrB*2 in *Salmonella* Typhimurium provides a slight advantage over *Salmonella* I 4,[5],12:i:- at a low concentration of enrofloxacin of 0.25 µg/ml and high concentration of 1 µg/ml.

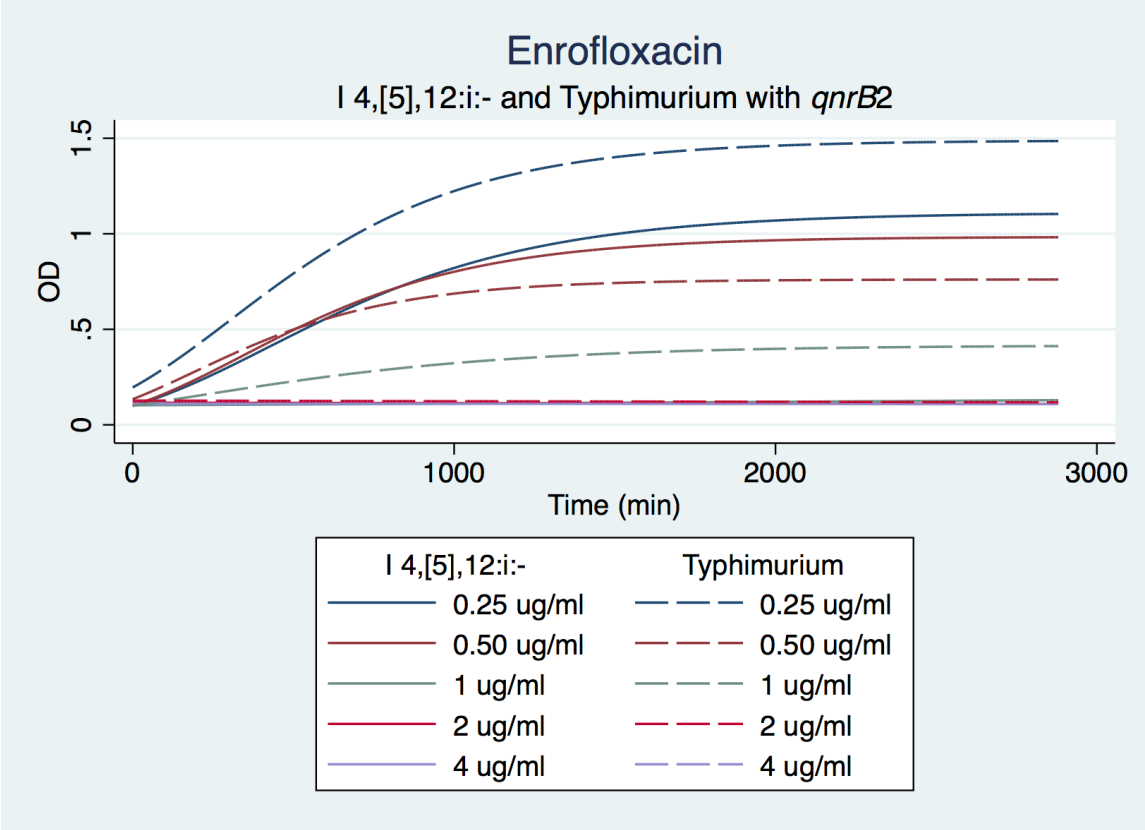


Figure 4-7 Fitness comparison of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium with *qnrB*2 at different enrofloxacin concentrations

4.3.1.2. Other monophasic Salmonella *serovars*

Only one *Salmonella* Derby isolate harbored the *qnrB*19 gene, which also contained a *parC* point mutation. Figure 4-8 shows there is no fitness cost for *Salmonella* Derby with the *qnrB*19 gene compared to the baseline, similarly to *Salmonella* I 4,[5],12:i:- with the *qnrB*19 gene. However, there is an increased fitness of Derby with the *qnrB*19 with no antibiotic present compared to the baseline. This may be due to the additional presence of the *parC* point mutation. Similar to *Salmonella* Typhimurium with *qnrB*2, *Salmonella* Derby with *qnrB19* (and *parC*) had a significant increase in fitness (increase in growth rate across all phases) at 0.25 µg/ml compared to when no enrofloxacin is present (Figure 4-8). Starting at 0.5 µg/ml of enrofloxacin, there was a significant reduction in growth which is further reduced at 1 µg/ml ($p \leq 0.05$). At 2 µg/ml, a similar growth curve to *Salmonella* I 4,[5],12:i:- with *qnrB*19 at 2 µg/ml is observed. However, there are major differences between these two serovars with the *qnrB*19 gene. *Salmonella* Derby with *qnrB*19 showed a shorter lag phase (before 1000 min), quicker exponential growth, and greater OD (OD > 0.5) at the stationary phase compared to *Salmonella* I 4,[5],12:i:- with the *qnrB*19 gene (OD ≤ 0.5). Additionally, at 4 µg/ml, growth is still observed for the *Salmonella* Derby isolate, whereas, for *Salmonella* I 4,[5],12:i:- with *qnrB*19 growth was inhibited.

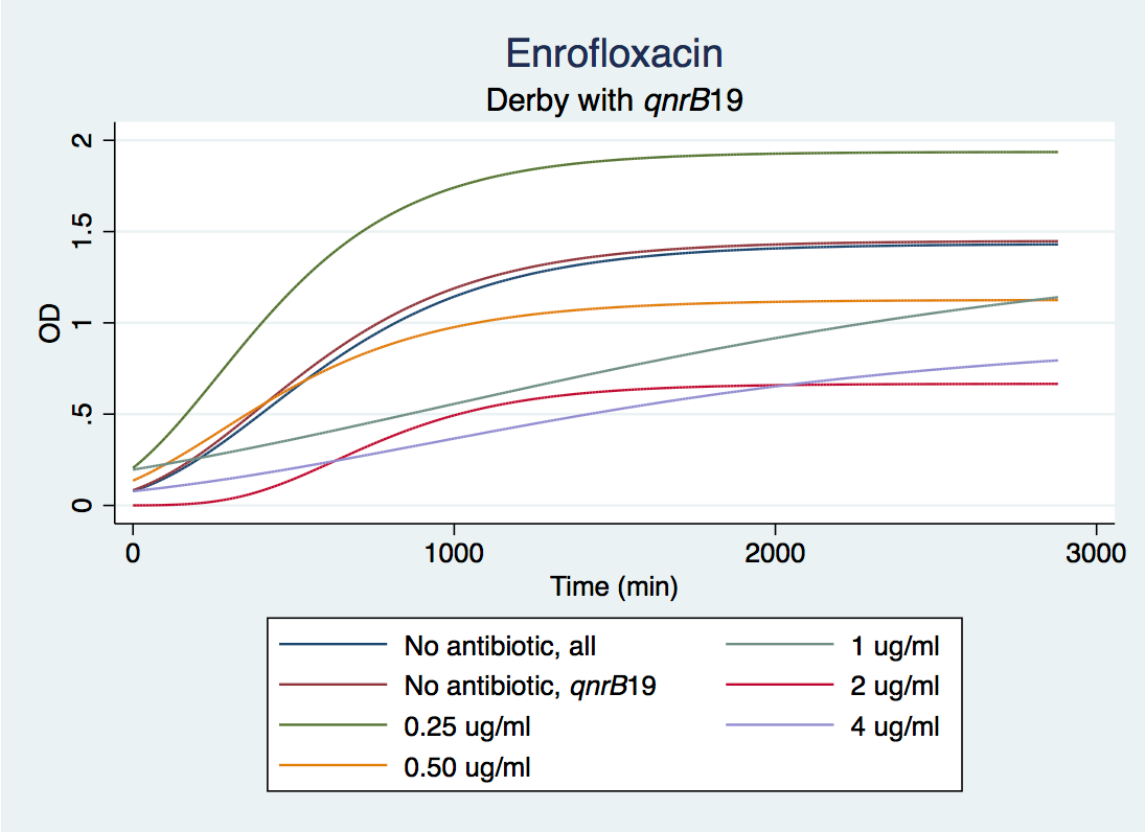


Figure 4-8 3-Parameter Gompertz fitted growth curves of *Salmonella* Derby with the *qnrB*19 gene at different enrofloxacin concentrations

There was one *Salmonella* Senftenberg isolate that harbored the *qnrB*19 gene. This isolate also contained a point mutation in the *parC* gene. Figure 4-9 shows that there is no fitness cost to harboring *qnrB*19 as there were no significant differences at the lag and exponential phase ($p \leq 0.05$) compared with the baseline. However, at the stationary phase the growth rate was greater than the baseline, most likely due to the additional point mutation of *parC*. Growth at 0.25 µg/ml was significantly greater than when no antibiotic

was present (Figure 4-9). At 0.5 µg/ml the growth rates decreased significantly at all phases; although, the lag phase takes longer than with no enrofloxacin and the exponential phase is slower, the growth rate at the stationary phase was significantly higher ($p \leq 0.05$). Thus, the combination of both the PMQR gene and the point mutation appears to enhance the ability of *Salmonella* Senftenberg to grow at low concentrations of enrofloxacin (0.25 and 0.50 µg/ml). The growth rate begins to decrease significantly at all phases ($p \leq 0.05$) after 1 µg/ml; however, growth was still observed at higher concentrations (2 and 4 µg/ml). This contrasts with *Salmonella* I 4,[5],12:i:- with *qnrB*19, which had a slower growth rate at low concentrations (1 and 2 µg/ml) and was unable to grow at 4 µg/ml. Growth rates for *Salmonella* Senftenberg with *qnrB19* and the *parC* mutation also tended to be greater than *Salmonella* Derby with *qnrB*19 and the *parC* mutation.

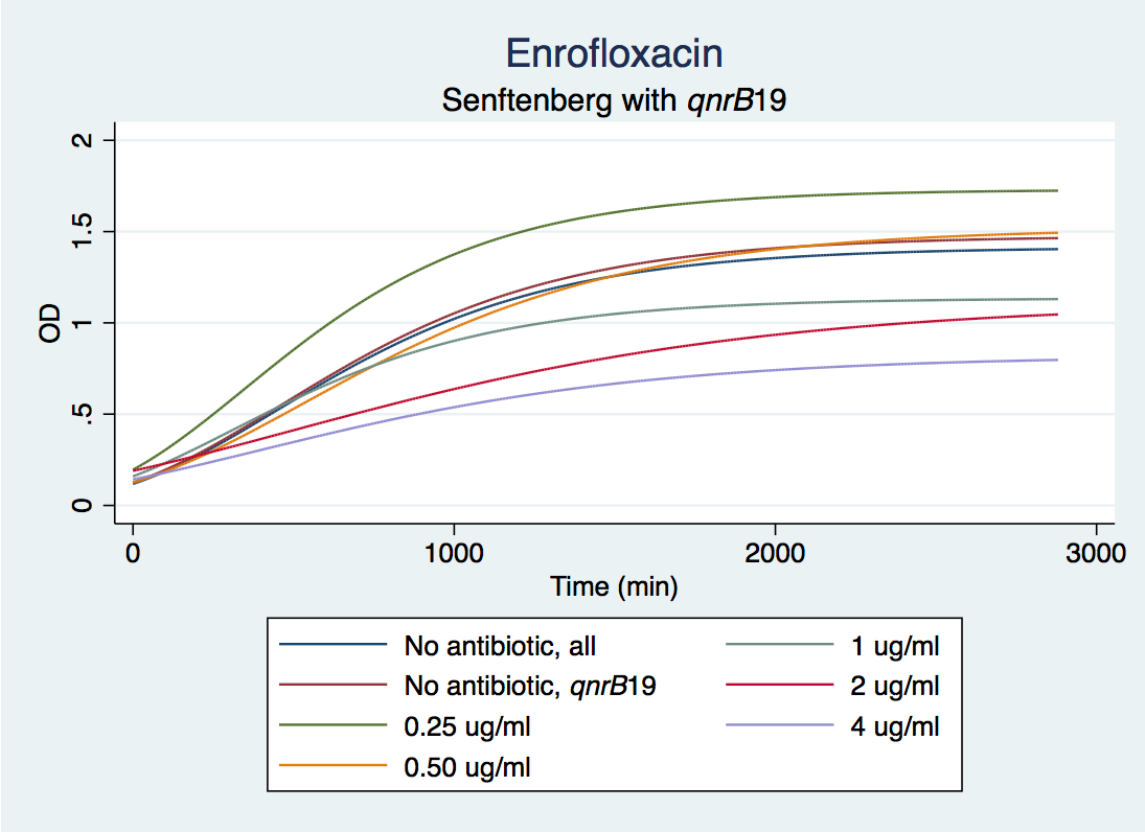


Figure 4-9 3-Parameter Gompertz fitted growth curves of *Salmonella* Senftenberg with the *qnrB*19 gene at different enrofloxacin concentrations

There were seven *Salmonella* Senftenberg isolates that harbored *qnrB*2, *aac(6')-Ib-cr*, and a *parC* point mutation. In the absence of enrofloxacin, there were no significant differences ($p \leq 0.05$) between the baseline and growth of *Salmonella* Senftenberg harboring the *qnrB*2 and *aac(6')-Ib-cr* at the lag and exponential phases. However, there seems to be a slight fitness cost associated with these genes at the stationary phase as there is a decrease in growth (Figure 4-10). The fitness cost may be attributed to the *qnrB*2 gene

as *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium showed reduced fitness (compared to the baseline) when the gene was present. Furthermore, the growth rate at the stationary phase is significantly greater ($p \leq 0.05$) at 0.25 and 0.5 µg/ml of enrofloxacin than with no antibiotic present (Figure 4-10). However, the exponential growth rate was slightly less than at baseline ($p \leq 0.5$). As the concentration of enrofloxacin increases to 1 and 2 ug/ml, the growth rate decreases significantly ($p \leq 0.05$) at all phases and growth is inhibited at 4 µg/ml. Harboring *qnrB*2, *aac(6')-Ib-cr*, as well as the *parC* point mutation allows *Salmonella* Senftenberg to grow at all concentrations of enrofloxacin except 4 µg/ml. Additionally, growth at 2 µg/ml was still observed from *Salmonella* Senftenberg in contrast to *Salmonella* I 4,[5],12:i:- (Figure 4-4) and *Salmonella* Typhimurium (Figure 4-6) with only the *qnrB*2 gene, which were inhibited by 2 µg/ml. At 2 µg/ml, the lag phase of *Salmonella* Senftenberg with *qnrB*2 was shorter than *Salmonella* I 4,[5],12:i:- with *qnrB*19. Although a slower exponential growth rate was observed for *Salmonella* Senftenberg (*qnrB*2, *aac(6')-Ib-cr*, and *parC* point mutation) than *Salmonella* I 4,[5],12:i:- with *qnrB*19, *Salmonella* Senftenberg had a greater growth rate once it reached the stationary phase compared to *Salmonella* I 4,[5],12:i:- with *qnrB*19 suggesting the presence of *qnrB*2, *aac(6')-Ib-cr*, and *parC* point mutation enhances its fitness at a later stage.

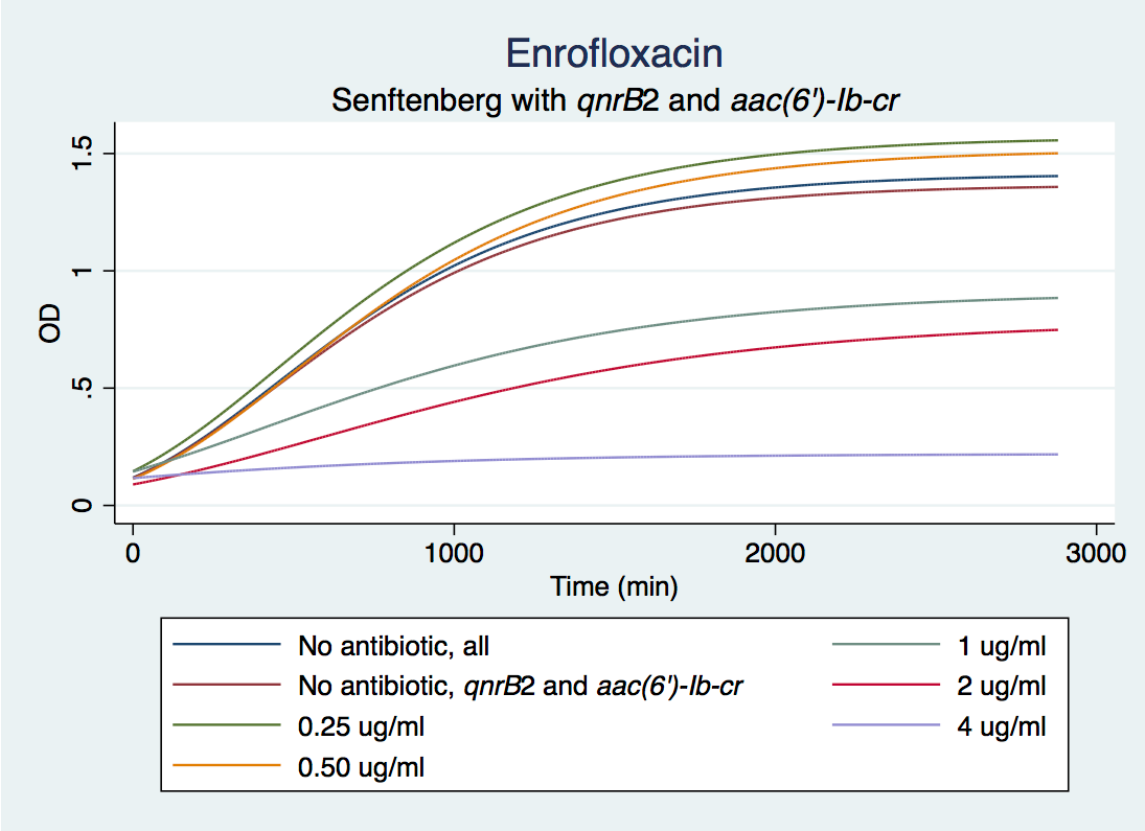


Figure 4-10 3-Parameter Gompertz fitted growth curves of *Salmonella* Senftenberg with the *qnrB*2 and *aac(6')-Ib-cr* gene at different enrofloxacin concentrations

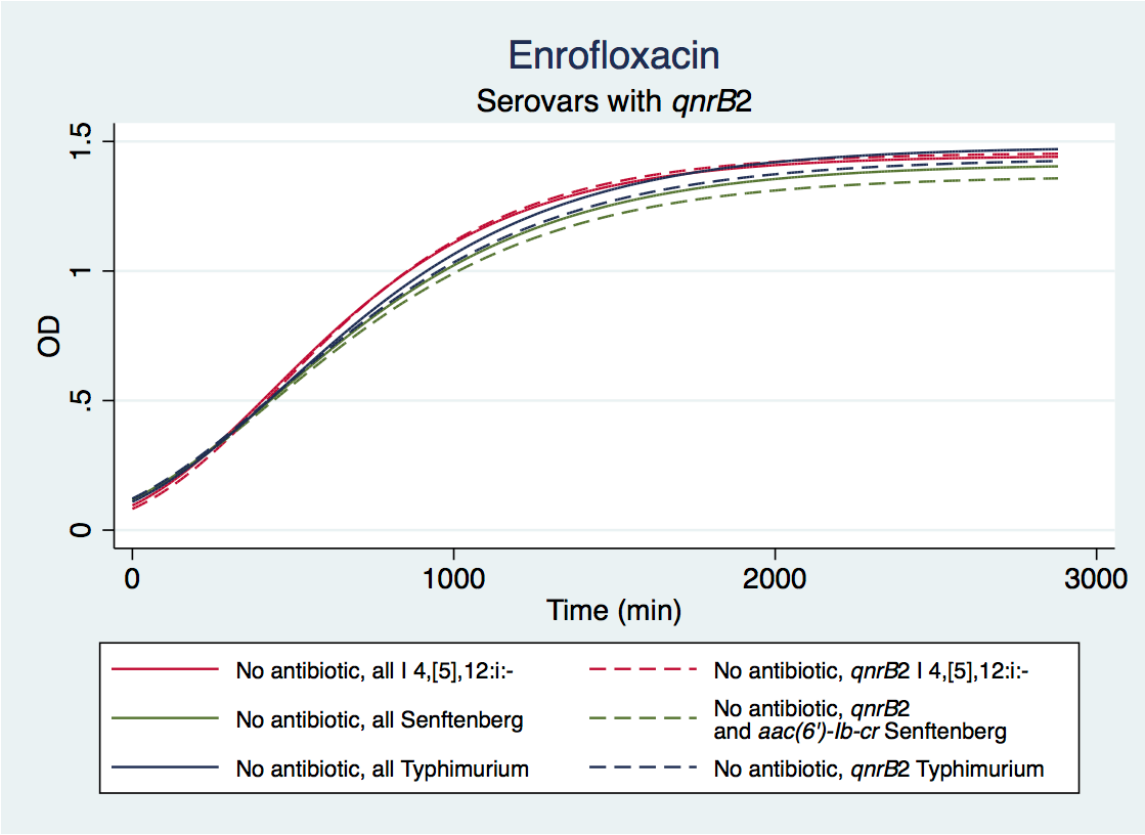


Figure 4-11 Fitness costs of *Salmonella* serovars with *qnrB*2

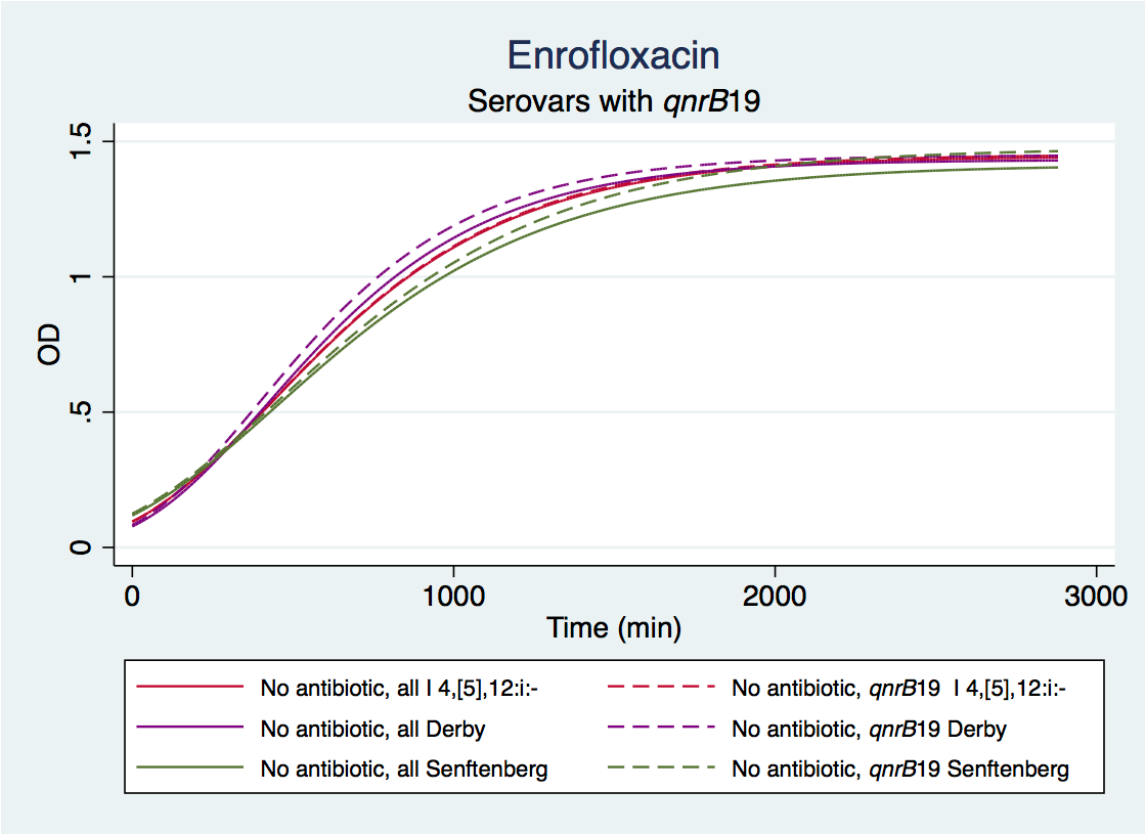


Figure 4-12 Fitness costs of *Salmonella* serovars with *qnrB*19

*4.3.2. Ceftiofur: beta-lactamase resistance gene (*bla$_{SHV-12}$*)*

4.3.2.1. Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

Salmonella I 4,[5],12:i:- harboring the *bla*$_{SHV-12}$ gene (n = 3) showed no fitness cost when compared to the baseline (Figure 4-13). The growth rate of *Salmonella* I 4,[5],12:i:- with *bla*$_{SHV-12}$ grown without enrofloxacin in the medium was slightly greater than the baseline at the lag and exponential phase, showing a slight increase in fitness ($p \leq 0.05$).

At 2 μg/ml, the growth rates at the exponential and stationary phase are significantly lower ($p \leq 0.05$) than at baseline (Figure 4-13). The growth of *Salmonella* I 4,[5],12:i:- with bla_{SHV-12} decreases as the concentrations of ceftiofur increased. Though there were slight differences at the lag and stationary phase between ceftiofur concentrations at 4, 6, and 8 μg/ml ($p \leq 0.05$), the exponential growth rate did not differ among the three concentrations. The lag phase was significantly longer at 8 μg/ml than at 4 μg/ml and the growth rate at the stationary phase was less at 8 μg/ml than at 4 μg/ml as expected. However, at 6 μg/ml, while the lag phase was the lowest compared to 4 and 8 μg/ml, the growth rate at the stationary phase was greater than at 4 and 8 μg/ml. The presence of bla_{SHV-12} in *Salmonella* I 4,[5],12:i:- strains increases its fitness at various concentrations of ceftiofur.

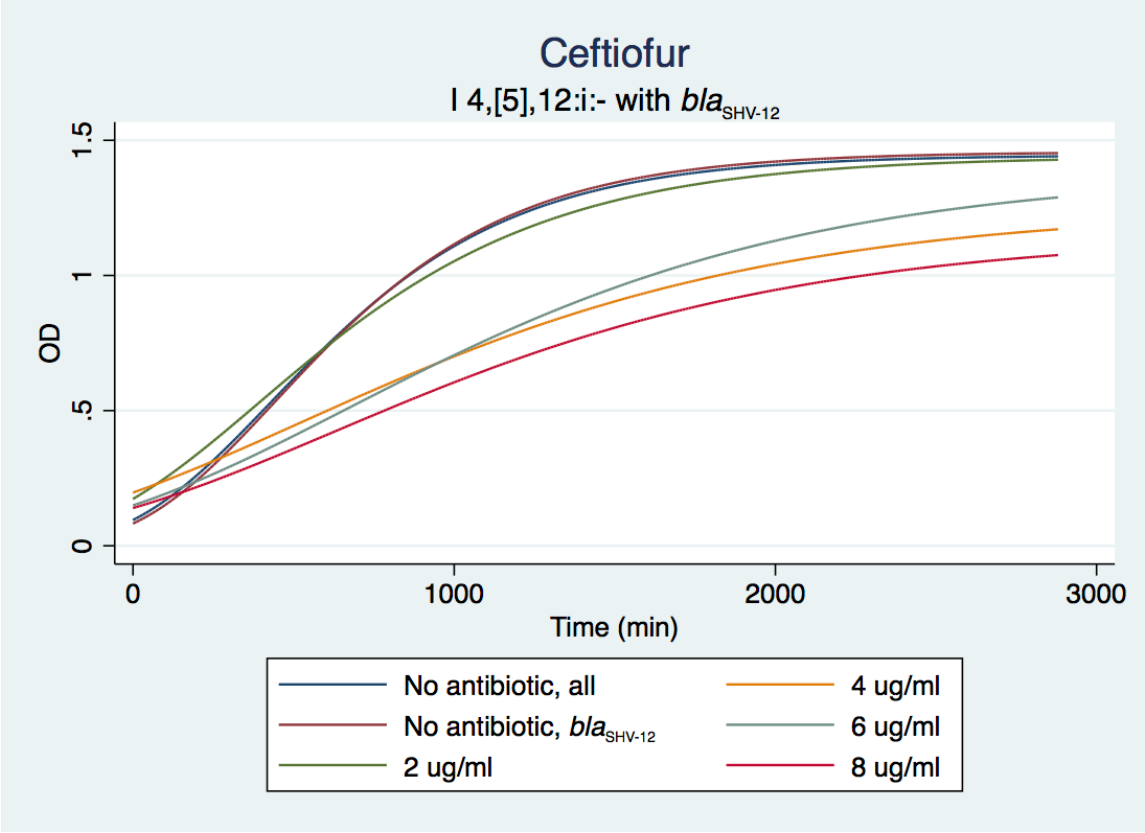


Figure 4-13 3-Parameter Gompertz fitted growth curves of *Salmonella* I 4,[5],12:i:- with the bla_{SHV-12} gene at different ceftiofur concentrations

In Figure 4-14, a fitness cost to harboring bla_{SHV-12} is observed in *Salmonella* Typhimurium isolates (n = 4) was observed, as there were significant differences ($p \leq 0.05$) with the baseline curve at all phases (slower growth rates). At the lowest concentration of ceftiofur (2 µg/ml), there is a slight decrease in the growth rate compared to the baseline ($p \leq 0.05$) (Figure 4-14). Once the concentration of the ceftiofur increased to 4 to 8 µg/ml, the growth rate decreased significantly at all phases ($p \leq 0.05$) and were

statistically different between each concentration ($p \leq 0.05$). The lag phase was significantly longer as the concentration increased. However, at the exponential phase, the growth rates marginally differed among the concentrations 4 to 8 µg/ml. At the stationary phase, the growth rate is significantly ($p \leq 0.05$) greater at 6 µg/ml of ceftiofur than 4 or 8 µg/ml as seen for *Salmonella* I 4,[5],12:i:- with $bla_{\text{SHV-12}}$. Furthermore, at 8 µg/ml, the growth rate at the stationary phase surpasses that of 4 µg/ml. *Salmonella* Typhimurium with $bla_{\text{SHV-12}}$ seem to have an increase in fitness at various concentrations of ceftiofur, particularly at higher concentrations (6 and 8 µg/ml).

When comparing *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- with $bla_{\text{SHV-12}}$, a fitness cost was observed in *Salmonella* Typhimurium strains while *Salmonella* I 4,[5],12:i:- had no fitness cost to harboring $bla_{\text{SHV-12}}$. Interestingly, *Salmonella* Typhimurium had shorter lag phases and greater exponential growth rates across all concentrations. However, by the stationary phase, the growth rates for *Salmonella* I 4,[5],12:i:- were greater at all concentrations of ceftiofur.

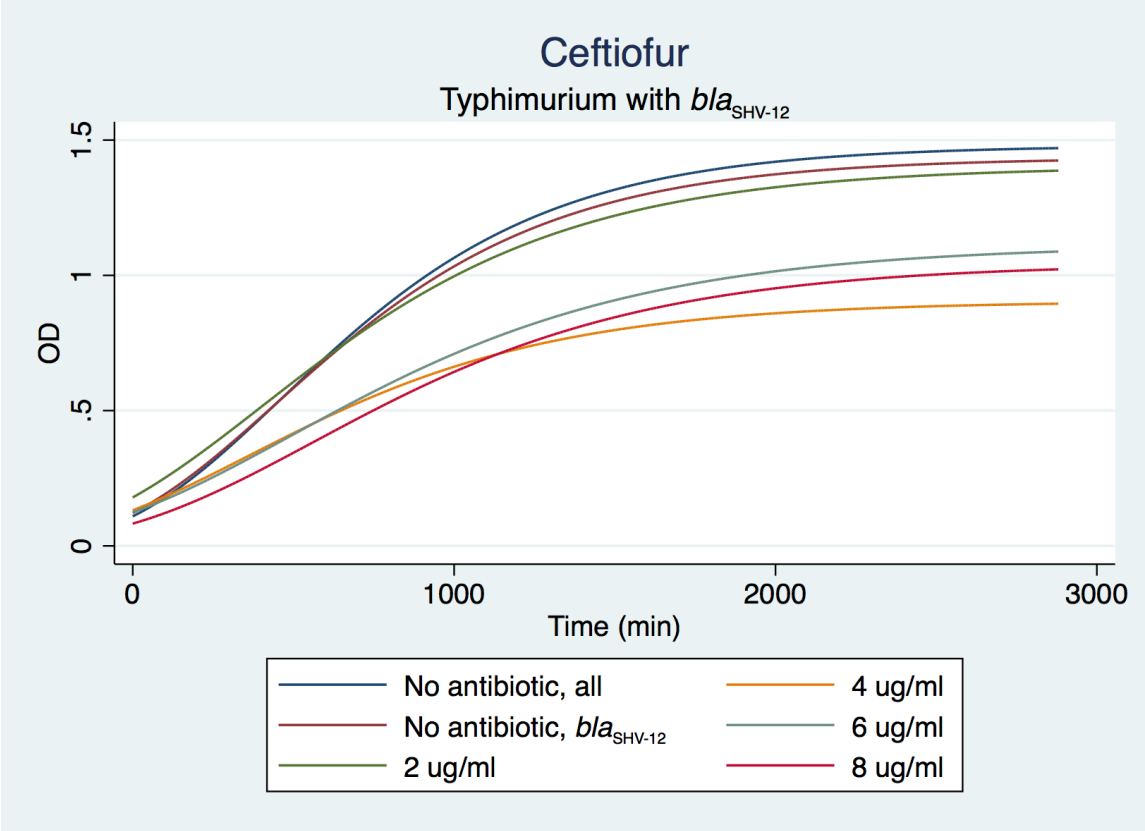


Figure 4-14 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the bla_{SHV-12} gene at different ceftiofur concentrations

4.3.2.2. Other monophasic Salmonella *serovars*

A small fitness cost was observed for *Salmonella* Senftenberg isolates harboring the bla_{SHV-12} gene (n = 7) at the stationary phase as it was significantly different than the baseline (Figure 4-15). As opposed to *Salmonella* serovars I 4,[5],12:i:- and Typhimurium, the presence of bla_{SHV-12} in *Salmonella* Senftenberg appears to be beneficial at 2 µg/ml of ceftiofur, as there was an increase in growth (Figure 4-15). Growth rate parameters at 4

and 6 µg/ml do not significantly differ from each other. However, the exponential growth rates begin to decrease significantly ($p \leq 0.05$) at 4 and 6 µg/ml compared to 2 ug/ml and is further reduced at 8 µg/ml of ceftiofur ($p \leq 0.05$). Additionally, the stationary phase is significantly less at 8 µg/ml than at other concentrations ($p \leq 0.05$). When compared to *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium with *bla*$_{SHV-12}$, *Salmonella* Senftenberg had greater exponential growth rates across all concentrations.

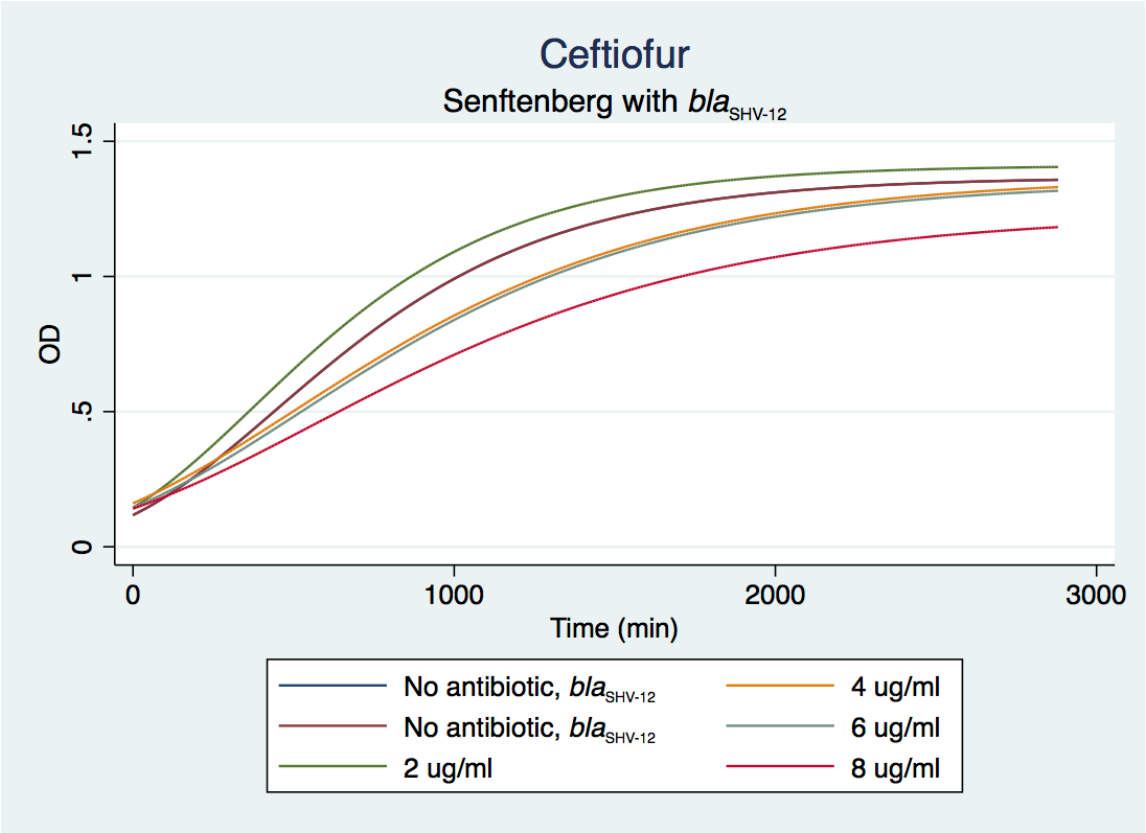


Figure 4-15 3-Parameter Gompertz fitted growth curves of *Salmonella* Senftenberg with the *bla*$_{SHV-12}$ gene at different ceftiofur concentrations

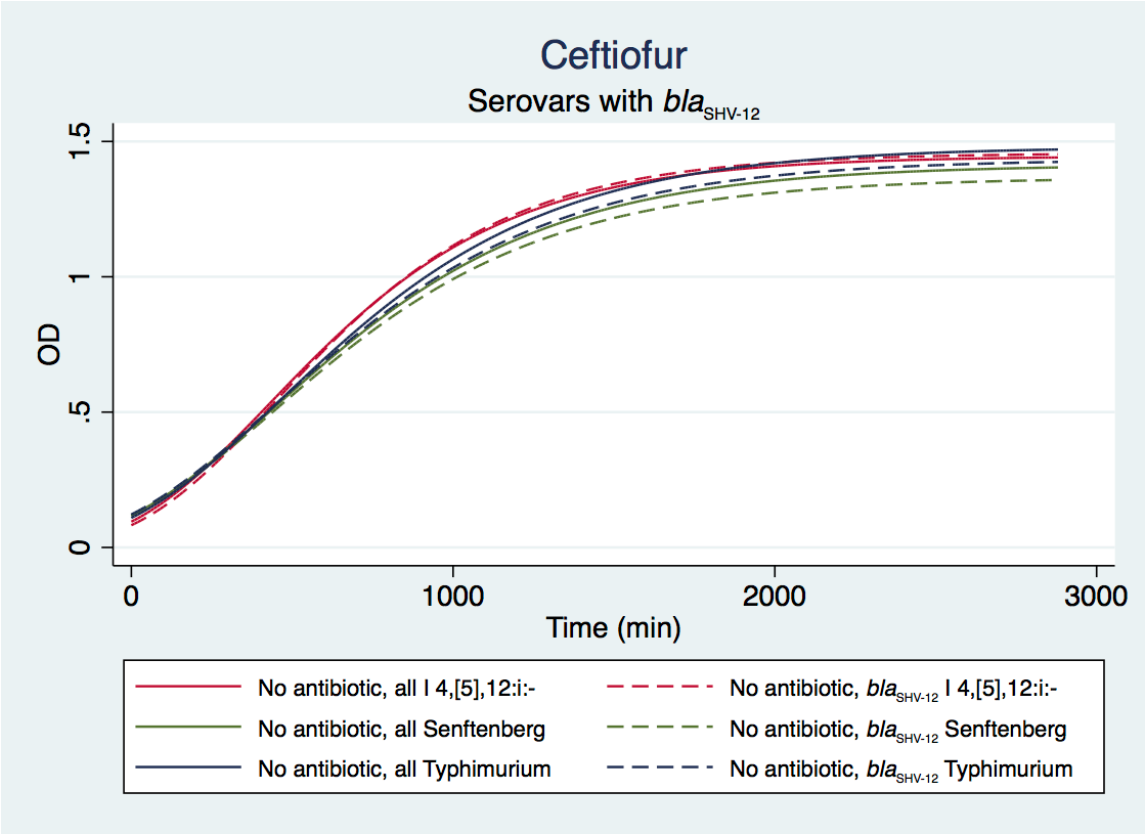


Figure 4-16 Fitness costs of *Salmonella* serovars with bla_{SHV-12}

*4.3.3. Tetracycline: tetracycline resistance genes (*tet*(A),* tet*(B),* tet*(D),* tet*(G))*

4.3.3.1. Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

All *Salmonella* I 4,[5],12:i:- harbored the *tet*(B) gene (n = 46) and were resistant to tetracycline. No fitness cost was observed to harboring the *tet*(B) when compared to the baseline (Figure 4-17). Although there were significant differences in growth rates at all phases across all tetracycline concentrations ($p \leq 0.05$), the differences were minor. The

lag phases were significantly ($p \leq 0.05$) longer and the growth rate at the exponential phase was significantly slower at all concentrations of tetracycline when compared to growth with no antibiotic. Additionally, the growth rates stabilized at the stationary phase and were not significantly different across tetracycline concentrations. One major difference was observed at 6 µg/ml, in which the growth rate was significantly slower at each phase compared to all other concentrations.

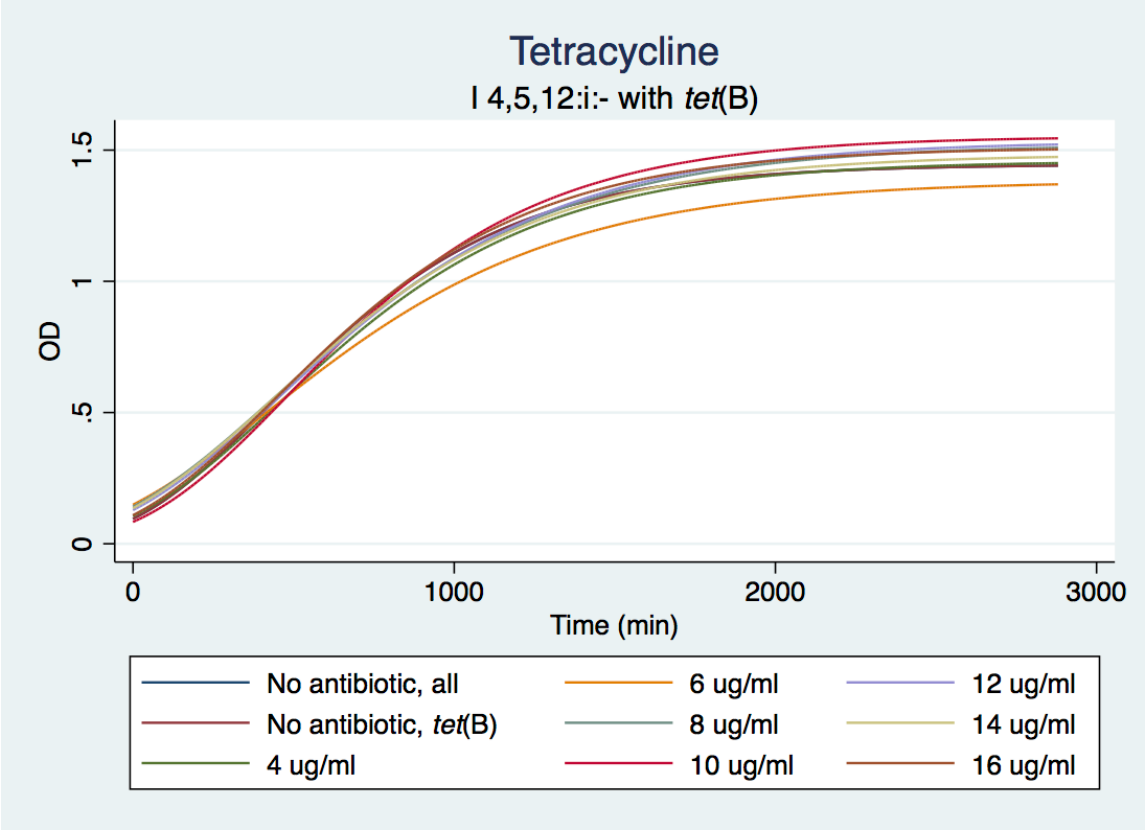


Figure 4-17 3-Parameter Gompertz fitted growth curves of *Salmonella* I 4,[5],12:i:- with the *tet*(B) gene at different tetracycline concentrations

Three of the 46 *Salmonella* I 4,[5],12:i:- isolates with *tet*(B) also harbored *tet*(D). As seen in Figure 4-18, there is no fitness cost to harboring the *tet*(B) and *tet*(D) genes when compared to the baseline. The addition of *tet*(D) increased the fitness of *Salmonella* I 4,[5],12:i:- as the growth rates were greater than the baseline at the exponential and stationary phase. Similarly, there were slight differences in growth rates with *Salmonella* I 4,[5],12:i:- harboring *tet*(B) and *tet*(D) across concentrations for all the growth parameters ($p \leq 0.5$). Also, the growth rate at 6 μg/ml was significantly slower at each phase compared to all other concentrations ($p \leq 0.05$). Furthermore, most of the differences were seen at the stationary phase, but growth rates varied and did not gradually decrease or increase as the concentration of tetracycline increased.

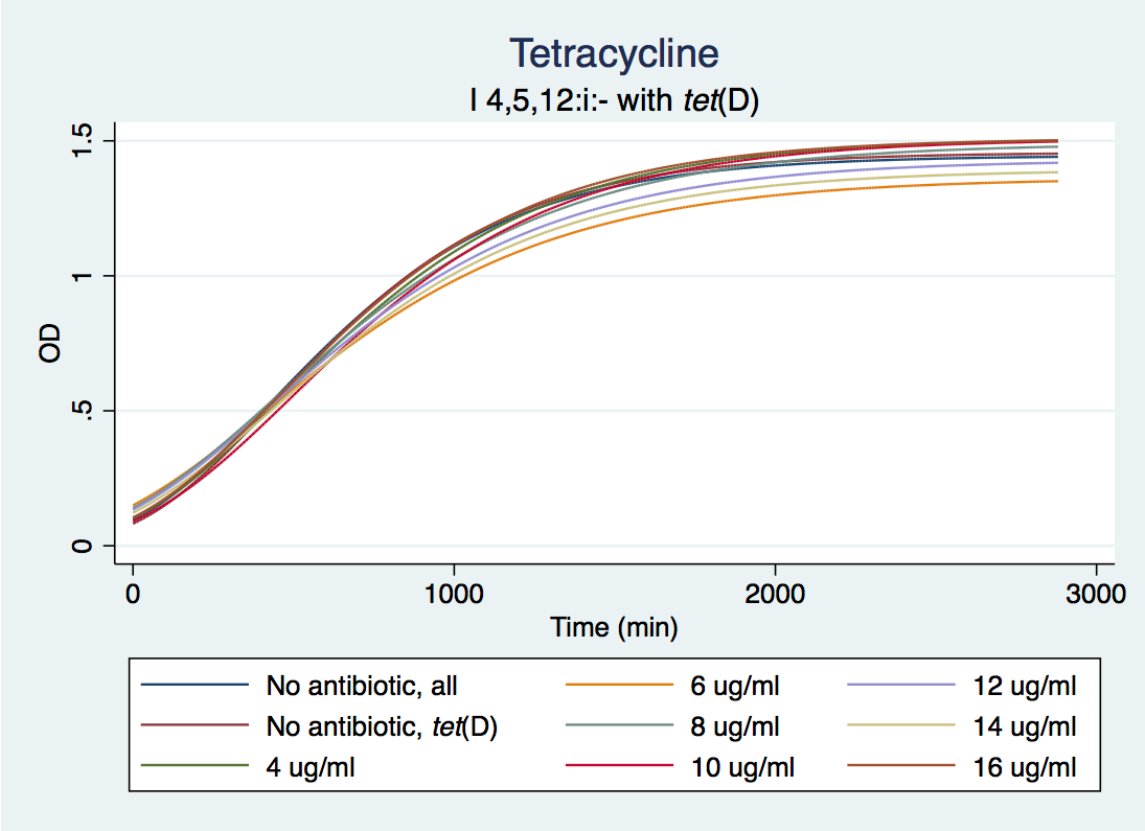


Figure 4-18 3-Parameter Gompertz fitted growth curves of *Salmonella* I 4,[5],12:i:- with the *tet*(D) and *tet*(B) gene at different tetracycline concentrations

Figure 4-19 shows there is no fitness cost to harboring *tet*(A) in *Salmonella* Typhimurium isolates (n = 13) when compared to the baseline. There were significant differences ($p \leq 0.05$) in growth rate all phase across tetracycline concentrations except at 12 μg/ml, which was significantly lower than at other concentrations. However, the main differences were seen at the stationary phase where growth rates varied and did not gradually decrease or increase as the concentration of tetracycline increased.

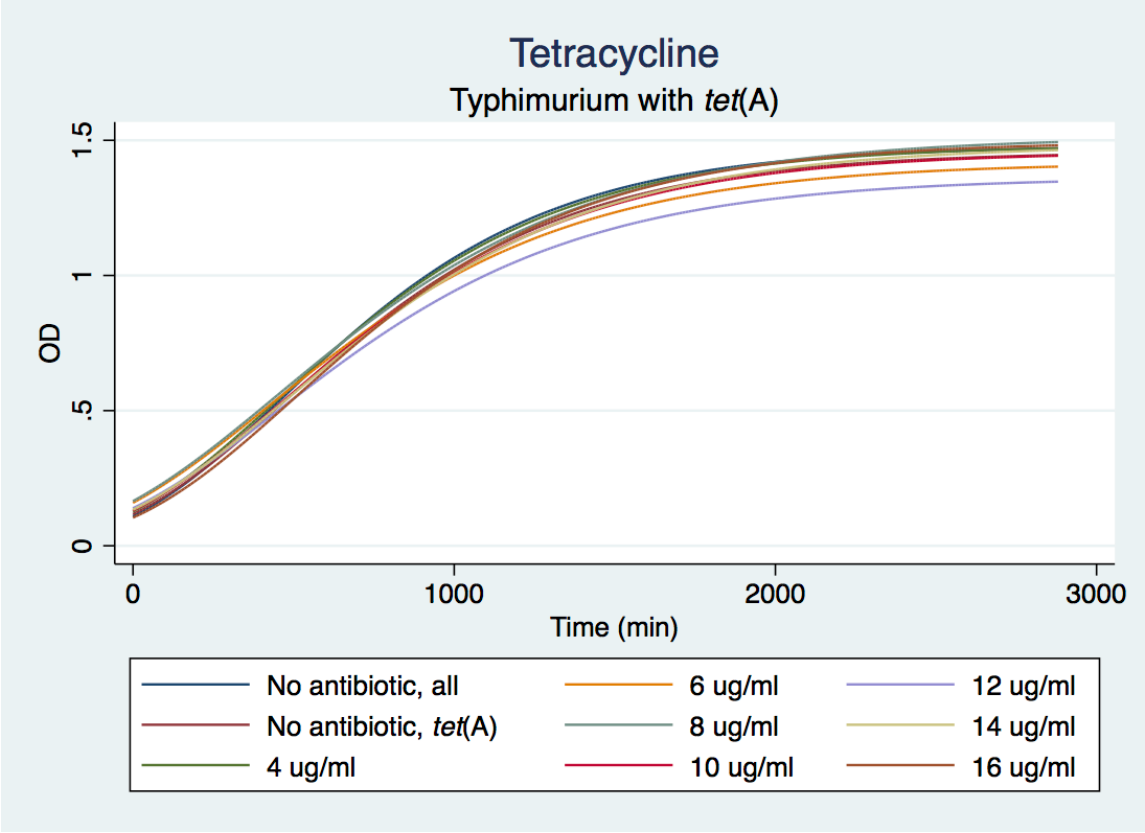


Figure 4-19 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the *tet*(A) gene at different tetracycline concentrations

Only one *Salmonella* Typhimurium isolate harboring *tet*(A) also harbored the *tet*(B) gene. There was a significant reduction ($p \leq 0.05$) in the growth rate at all phases of the *Salmonella* Typhimurium isolate harboring both the *tet*(A) and *tet*(B) genes (no antibiotic) when compared to the baseline (Figure 4-20). There was also a significant decrease in growth at all tetracycline concentrations compared to when tetracycline was

absent. Because there was no fitness cost to harboring *tet*(A), the fitness cost may be attributed to the additional *tet*(B) gene as seen in *Salmonella* I 4,[5],12:i:- isolates.

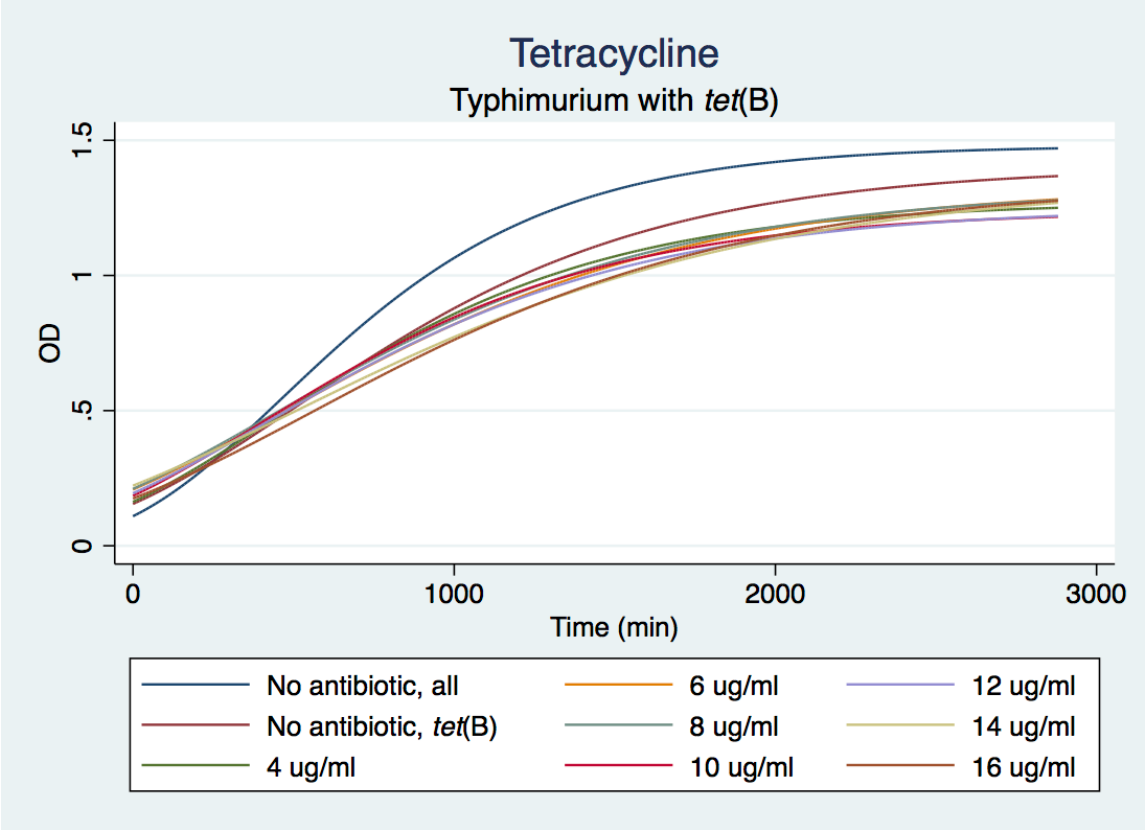


Figure 4-20 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the *tet*(B) gene at different tetracycline concentrations

Unlike *Salmonella* I 4,[5],12:i:- with *tet*(D), *Salmonella* Typhimurium had a fitness cost to harboring *tet*(D) (n = 4) grown with no antibiotic compared to the baseline ($p \leq 0.05$) (Figure 4-21). Furthermore, the growth rates of *Salmonella* Typhimurium with *tet*(D) at the lag and stationary phase begin to decrease significantly at 8 µg/ml and after 4 and 6 µg/ml of tetracycline ($p \leq 0.05$). *Salmonella* Typhimurium had slower growth rates

at the exponential phase (across all concentrations), but shorter lag phases and greater stationary growth rates than *Salmonella* I 4,[5],12:i:- with *tet*(D) or *tet*(B). Compared to *Salmonella* Typhimurium with *tet*(A), *Salmonella* Typhimurium with *tet*(D) had quicker exponential growth rates and slower lag times across all concentrations (except 16 µg/ml), but no differences were observed at the stationary phase.

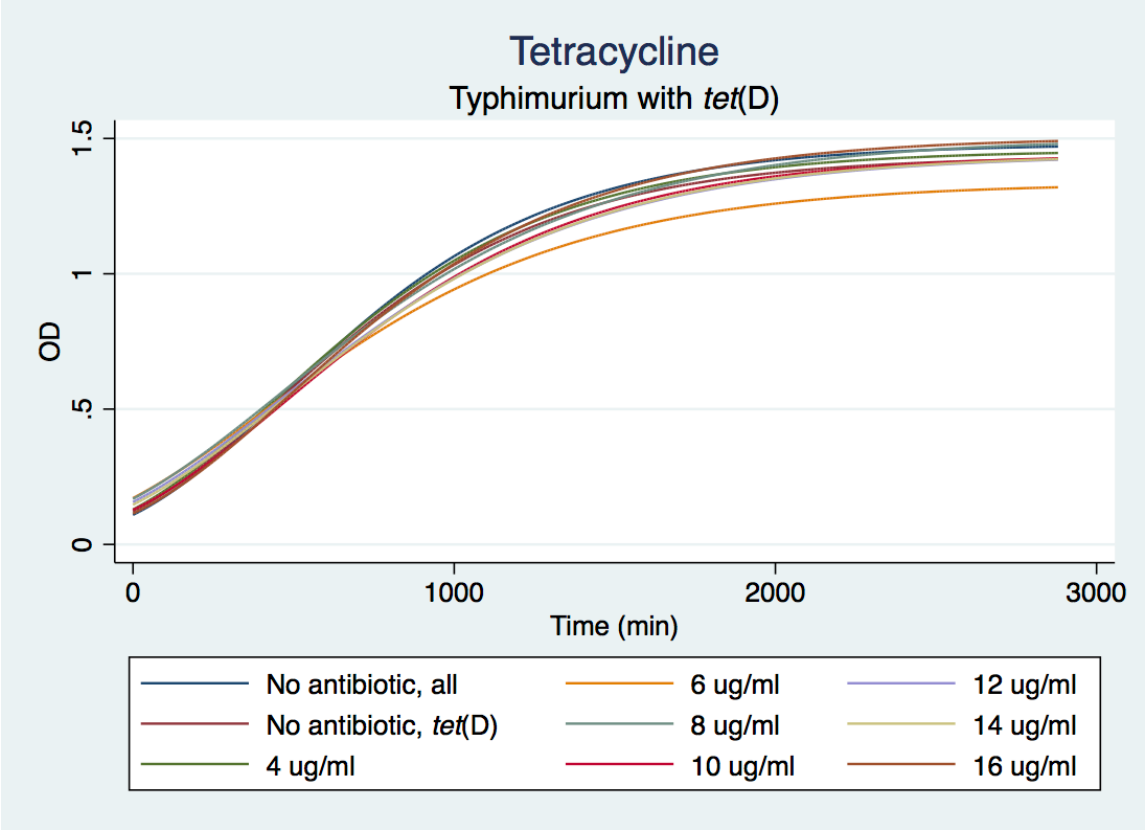


Figure 4-21 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the *tet*(D) gene at different tetracycline concentrations

No fitness cost was observed for *Salmonella* Typhimurium harboring *tet*(G) (n = 11). However, there was increased fitness compared to the baseline as seen in Figure 4-

22. *Salmonella* Typhimurium harboring *tet*(G) had much greater growth rates at all phases with no antibiotic and at all tetracycline concentrations than the baseline ($p \leq 0.5$). As seen in Figure 4-22, the lag phases were longer as the concentration of tetracycline increased and were significantly different ($p \leq 0.5$). Although *Salmonella* Typhimurium with *tet*(G), had longer lag phases than *Salmonella* Typhimurium or I 4,[5],12:i:- with other tetracycline resistance genes (*tet*(A), *tet*(B), or *tet*(D)), the exponential and stationary growth rates were greater. This suggests *tet*(G) improves the fitness of *Salmonella* Typhimurium more than other *tet* genes.

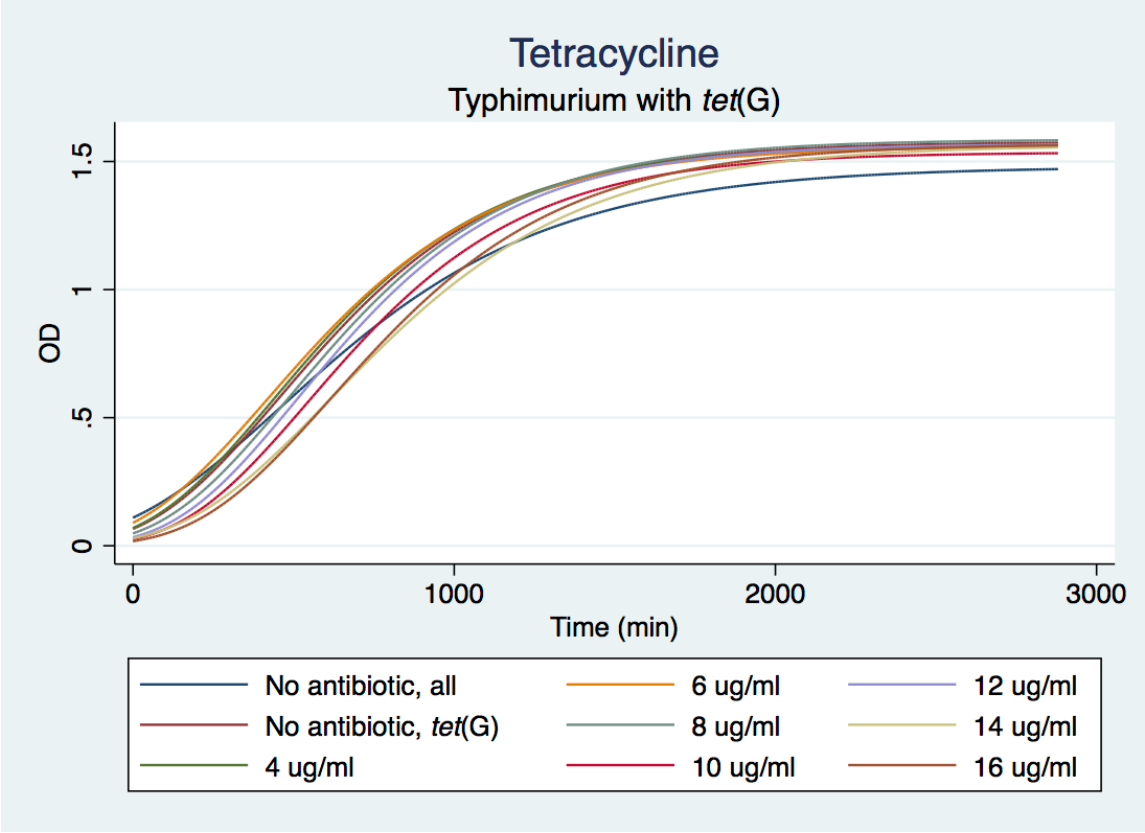


Figure 4-22 3-Parameter Gompertz fitted growth curves of *Salmonella* Typhimurium with the *tet*(G) gene at different tetracycline concentrations

4.3.3.2. Other monophasic Salmonella *serovars*

Salmonella Agona did not show a fitness cost to harboring *tet*(A) (n = 7) (Figure 4-23). Between 4 and 8 µg/ml of tetracycline, no significant differences were seen at the lag phase, while the exponential growth rates significantly increased ($p \leq 0.5$) (Figure 4-23). However, as the concentrations increased above 10 µg/ml, the exponential growth rate was significantly greater, the lag phase was significantly longer, and the stationary phase was significantly less than at 4 to 8 µg/ml ($p \leq 0.5$). The exponential growth rates

and stationary phases did not differ at tetracycline concentrations of 10, 12, and 14 µg/ml. All phases were significantly less at 16 µg/ml of tetracycline compared to all other concentrations ($p \leq 0.05$).

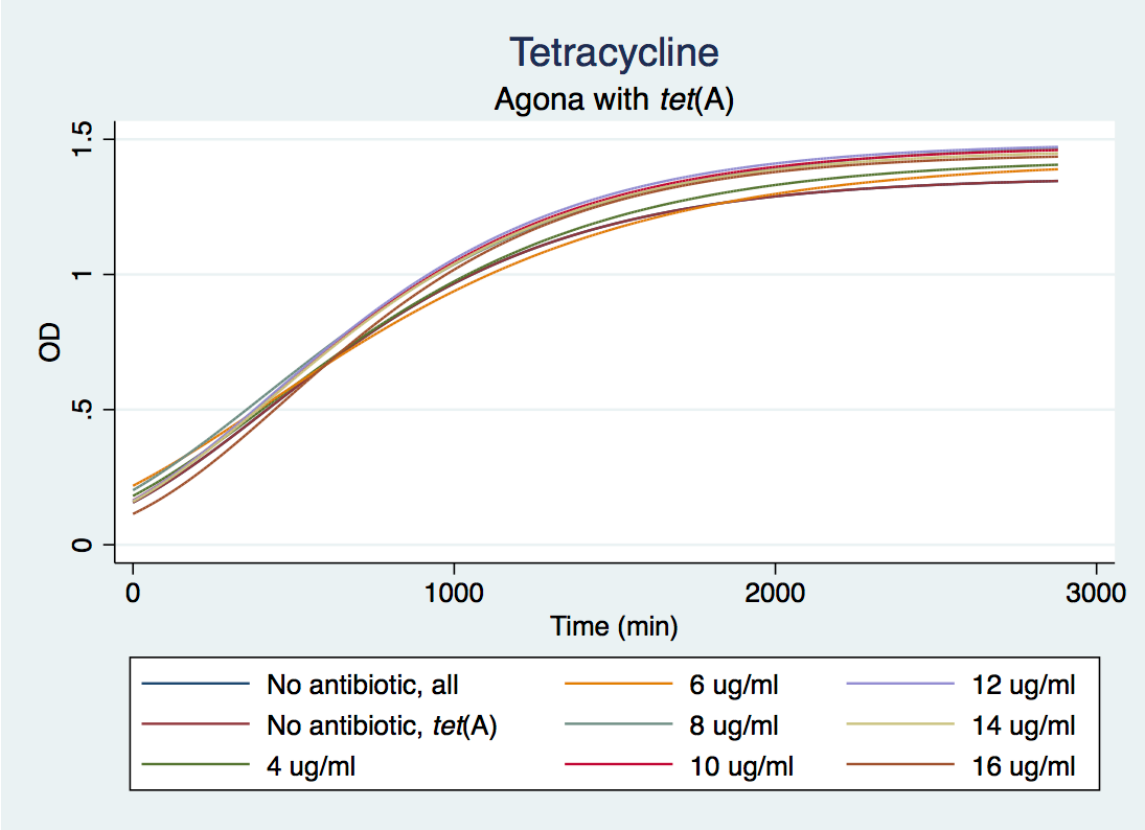


Figure 4-23 3-Parameter Gompertz fitted growth curves of *Salmonella* Agona with *tet*(A) gene at different tetracycline concentrations

Of the seven *Salmonella* Agona isolates harboring *tet*(A), one also harbored *tet*(B). Figure 4-24 shows a small fitness cost to harboring both *tet*(A) and *tet*(B) ($p \leq 0.05$), although it may be attributed to *tet*(B) as there was no fitness cost when only *tet*(A) was

present (Figure 4-23). The exponential growth rates were greater with the presence of both *tet* genes from 4 to 8 μg/ml than with only *tet*(A), however at the stationary phase the growth rate is significantly less ($p \leq 0.05$) (Figure 4-24). At higher concentrations of tetracycline (10 to 16 μg/ml), the opposite occurs where the exponential growth rates are significantly less with the presence of both *tet* genes and greater at the stationary phase ($p \leq 0.5$). Thus, although there is a minor fitness cost and reduced exponential growth rate to harboring both the *tet*(A) and *tet*(B) genes in *Salmonella* Agona (no antibiotic), the presence of both genes allowed growth at higher concentrations of tetracycline (stationary phases).

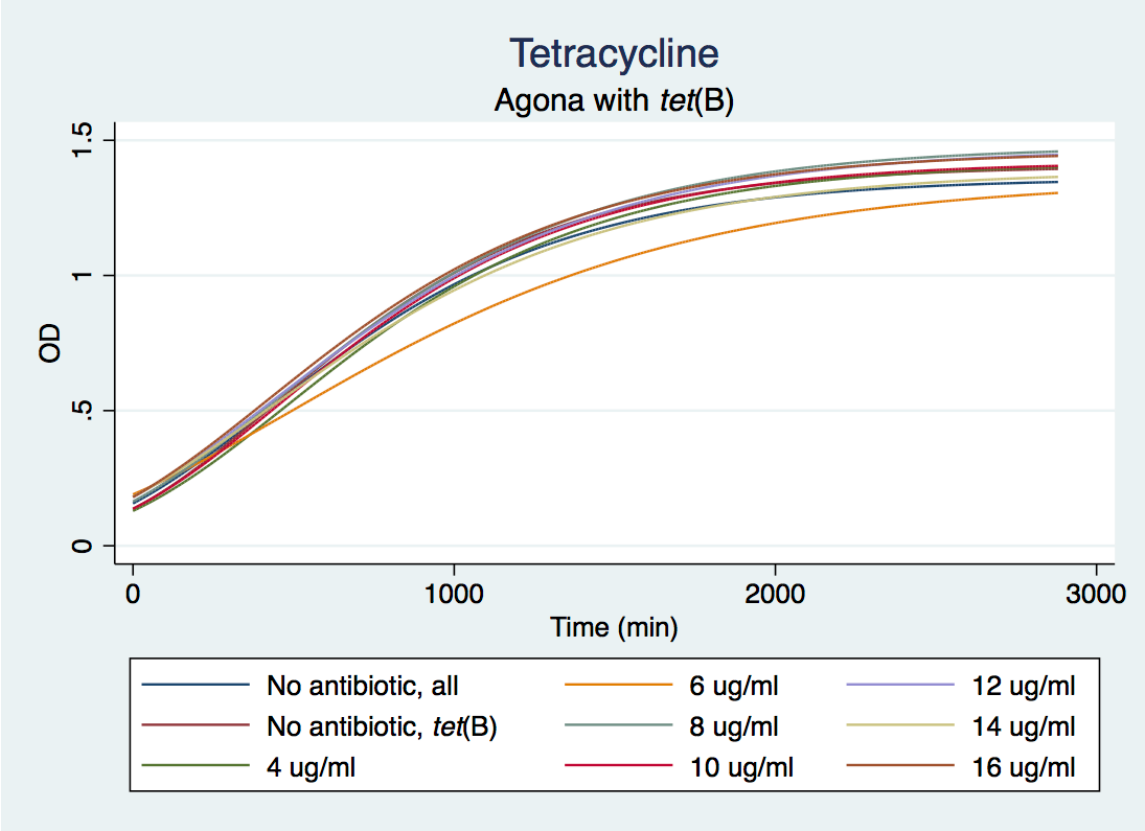


Figure 4-24 3-Parameter Gompertz fitted growth curves of *Salmonella* Agona with *tet*(A) and *tet*(B) gene at different tetracycline concentrations

Salmonella Alachua did not seem to have a fitness cost to harboring both *tet*(A) and *tet*(B) (Figure 4-25). Because all *Salmonella* Alachua isolates (n = 4) harbored both genes, we are unable to determine if an individual *tet* genes had a fitness cost as seen with *Salmonella* Typhimurium isolates harboring only *tet*(A) versus the *Salmonella* Typhimurium with both *tet*(A) and *tet*(B). The lag phase was longer for *Salmonella* Alachua with both *tet*(A) and *tet*(B) than *Salmonella* Typhimurium with *tet*(A) or both, but similar to *Salmonella* I 4,[5],12:i:- with *tet*(B). However, *Salmonella* Alachua was

observed to have a greater exponential growth rate, but a slower stationary phase (across all tetracycline concentrations) than *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- with the respective *tet* genes (Figure 4-25). Thus, *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- grew better during the lag phase and as they reach the stationary phase.

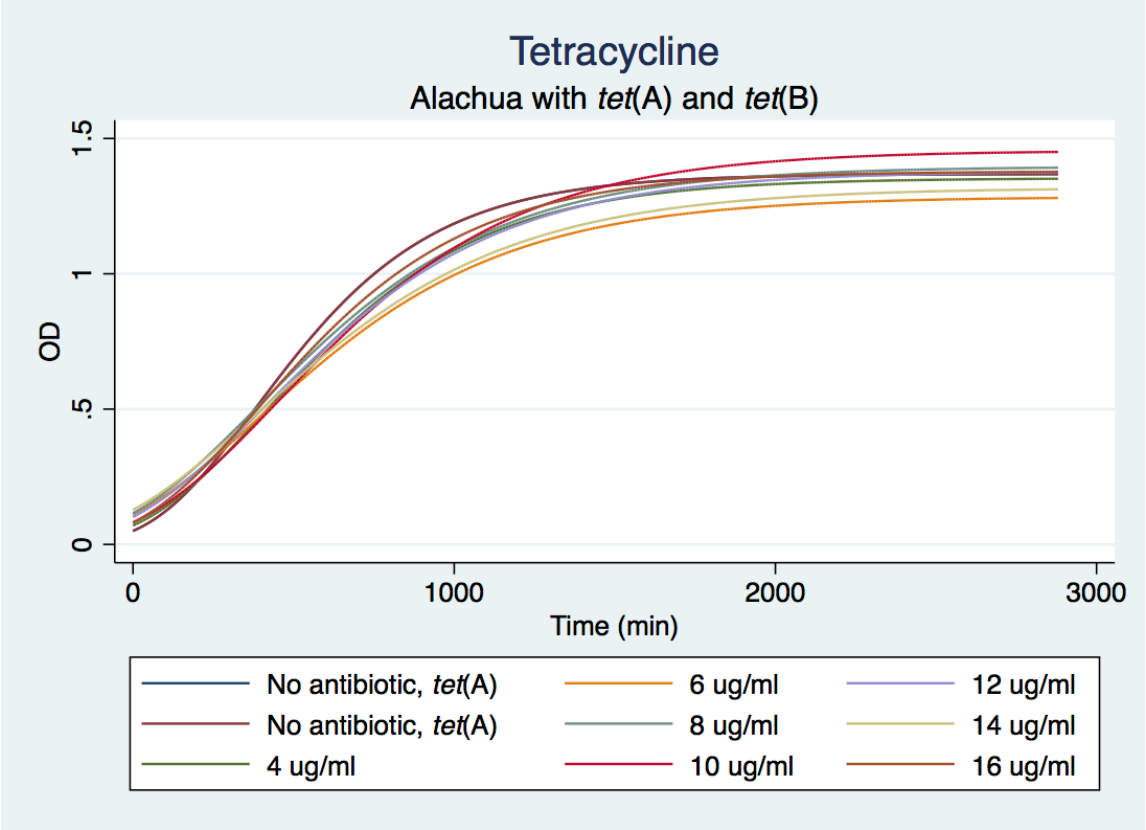


Figure 4-25 3-Parameter Gompertz fitted growth curves of *Salmonella* Alachua with *tet*(A) and *tet*(B) gene at different tetracycline concentrations

No fitness cost was observed in *Salmonella* Derby isolates harboring the *tet*(A) gene (n = 5) (Figure 4-26). While there are no significant differences between the

exponential growth rates and stationary phase, the lag phases become significantly longer as the tetracycline concentrations increase ($p \leq 0.05$) (Figure 4-26). However, *Salmonella* Derby with *tet*(A) had shorter lag phases as well as greater exponential and stationary phases than *Salmonella* serovars Typhimurium and *Salmonella* Agona with only *tet*(A).

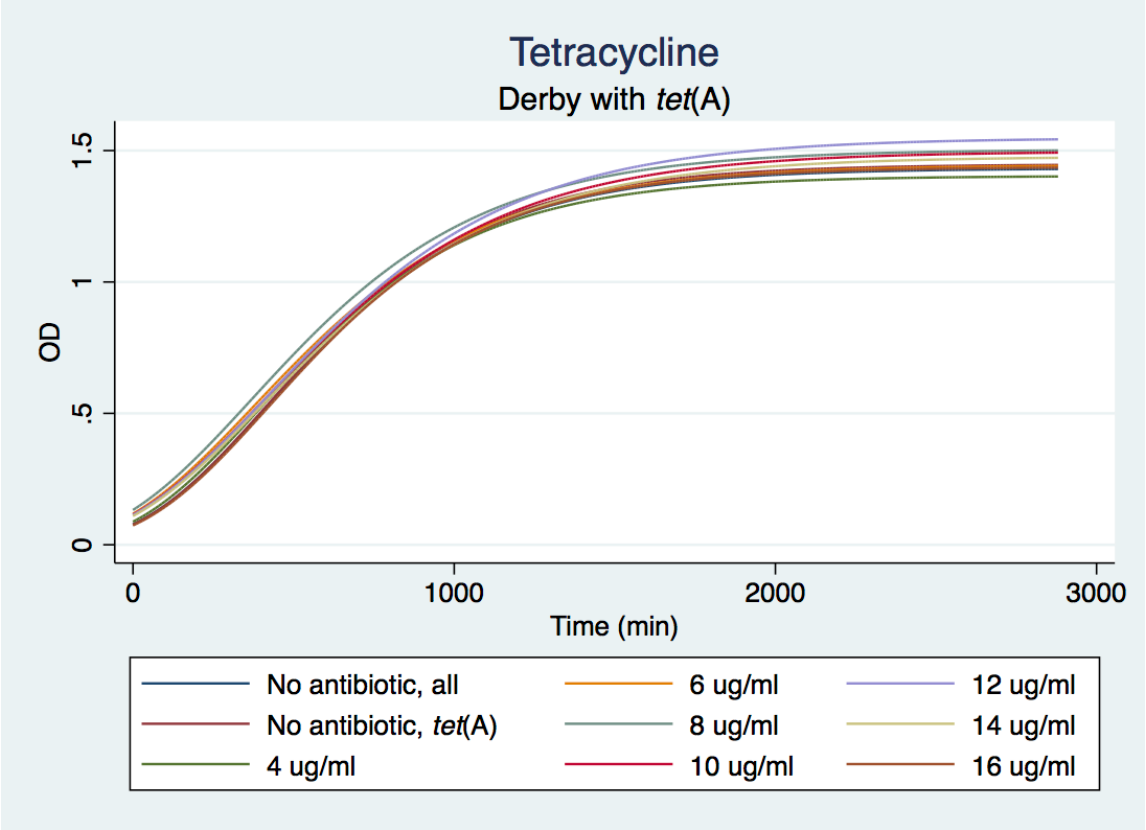


Figure 4-26 3-Parameter Gompertz fitted growth curves of *Salmonella* Derby with *tet*(A) gene at different tetracycline concentration

There was only one *Salmonella* Derby isolate harboring only the *tet*(B) gene. Unlike *Salmonella* Derby with *tet*(A) and similarly to other *Salmonella* serovars with *tet*(B), *Salmonella* Derby had a fitness cost in harboring *tet*(B) (Figure 4-27). As seen with

Salmonella Derby harboring *tet*(A), no significant differences were observed between the exponential growth rates and stationary phase as the tetracycline concentrations increase; however, the lag phases become significantly longer ($p \leq 0.05$) (Figure 4-27). Additionally, when compared to *Salmonella* Derby with *tet*(A), the growth rates at the stationary phase (across all concentrations) were less in *Salmonella* Derby with *tet*(B). Furthermore, although *Salmonella* Derby with *tet*(B) had shorter lag phases and greater exponential growth rates, the stationary growth rates of *Salmonella* I 4,[5],12:i:- with *tet*(B) were less.

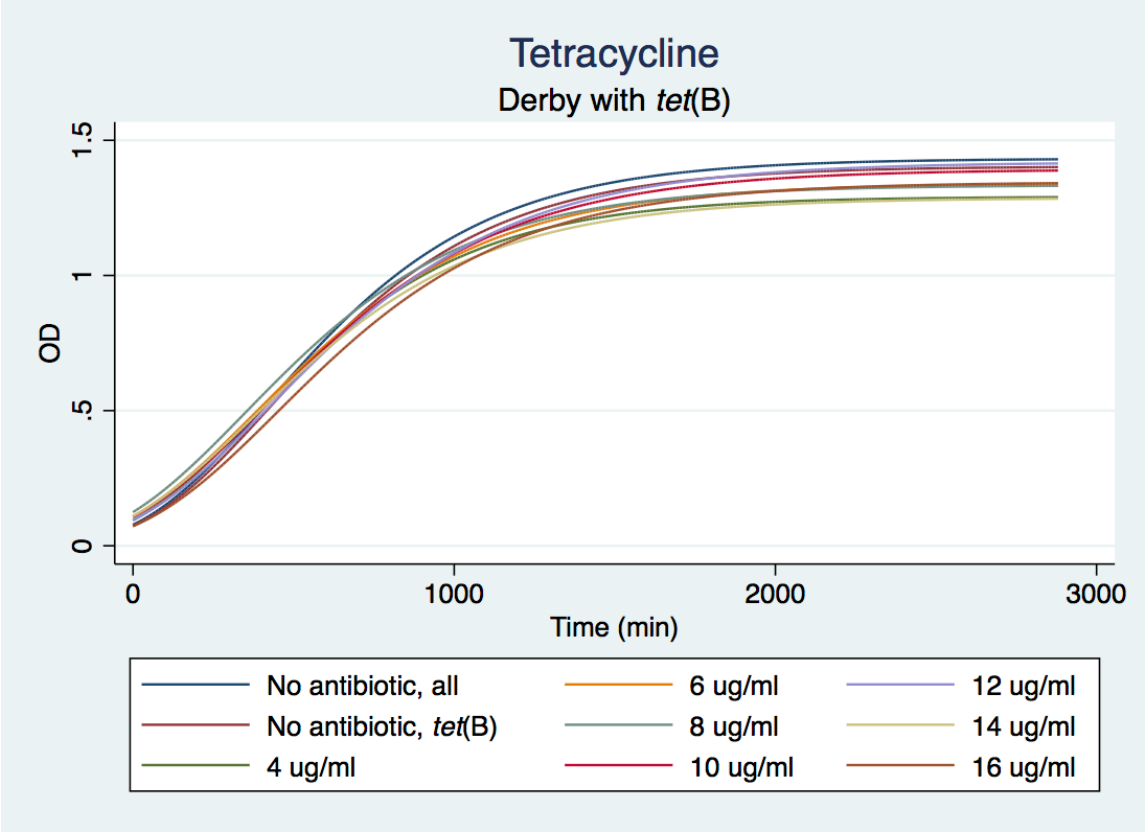


Figure 4-27 3-Parameter Gompertz fitted growth curves of *Salmonella* Derby with *tet*(B) gene at different tetracycline concentrations

The fitted growth curves in Figure 4-28 are of *Salmonella* Senftenberg harboring the *tet*(A) gene (n = 4); half also harbor the *tet*(D) gene. No fitness cost was observed for *Salmonella* Senftenberg harboring the *tet*(A)/*tet*(D) combination while there was a small cost at the stationary phase of *Salmonella* Senftenberg harboring *tet*(A) (Figure 4-28 and Figure 4-29). Figure 4-28 shows growth rates of *Salmonella* Senftenberg with only *tet*(A) do not significantly differ at all the phases between 4 and 6 µg/ml of tetracycline. However, in comparison to growth rates between 10 and 16 µg/ml, the lag phase is

significantly longer, and the exponential rate is significantly greater ($p \leq 0.05$). Despite differences between the lag and exponential phases, there were no significant differences at the stationary phase across between 10 and 16 µg/ml concentrations.

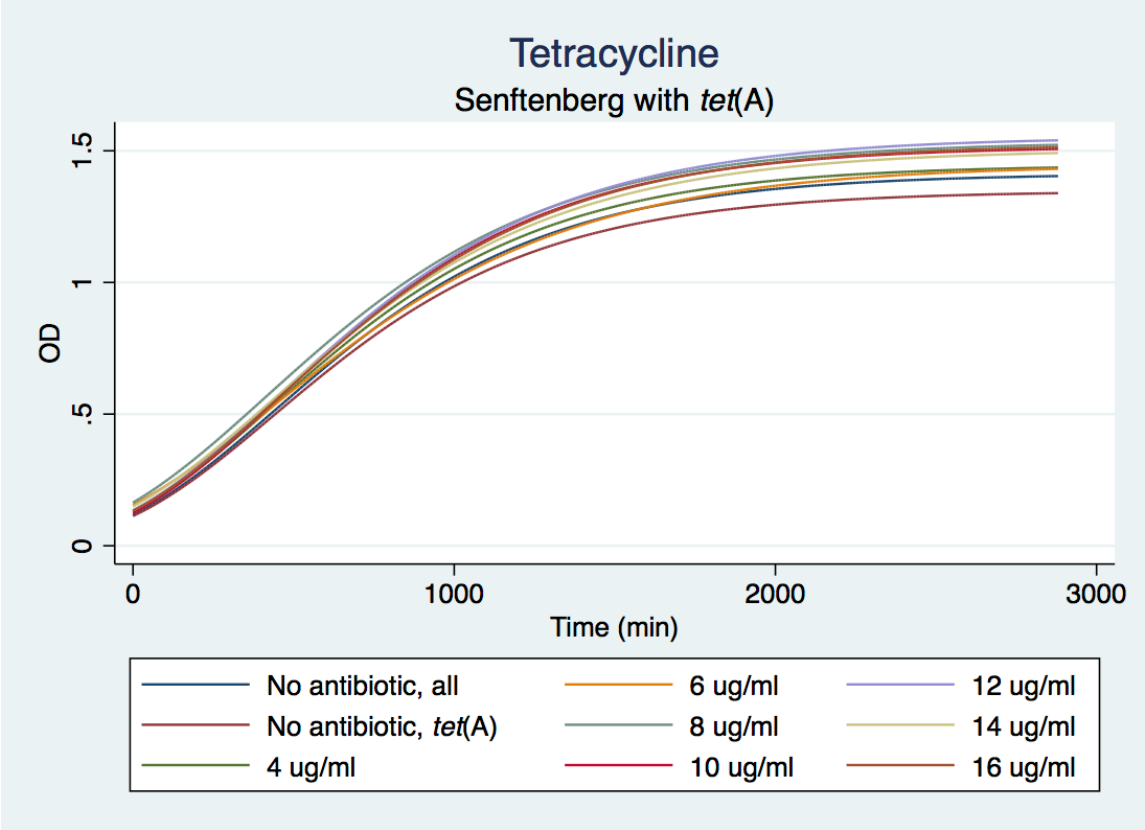


Figure 4-28 3-Parameter Gompertz fitted growth curves of *Salmonella* Senftenberg with *tet*(A) gene at different tetracycline concentrations

Figure 4-29 shows the fitted growth curves of *Salmonella* Senftenberg harboring *tet*(D) (including the two that harbor the *tet*(A) gene, as mentioned above). Although there was a minor reduction in growth rates at all phases, there were no significant differences in growth rates between *Salmonella* Senftenberg harboring *tet*(D) and the baseline, similar to *Salmonella* I 4,[5],12:i:- with *tet*(D). The lag phases become significantly longer as tetracycline increases from 4 to 12 μg/ml ($p \leq 0.05$), though at 14 and 16 μg/ml there are no significant differences compared to 6 μg/ml. The exponential growth rates of *Salmonella* Senftenberg with *tet*(D) significantly differ across all tetracycline concentrations ($p \leq 0.05$). When compared to *Salmonella* I 4,[5],12:i:- and Typhimurium with *tet*(D), *Salmonella* Senftenberg with *tet*(D) were observed to have significant differences at the lag and stationary phases (across all concentrations).

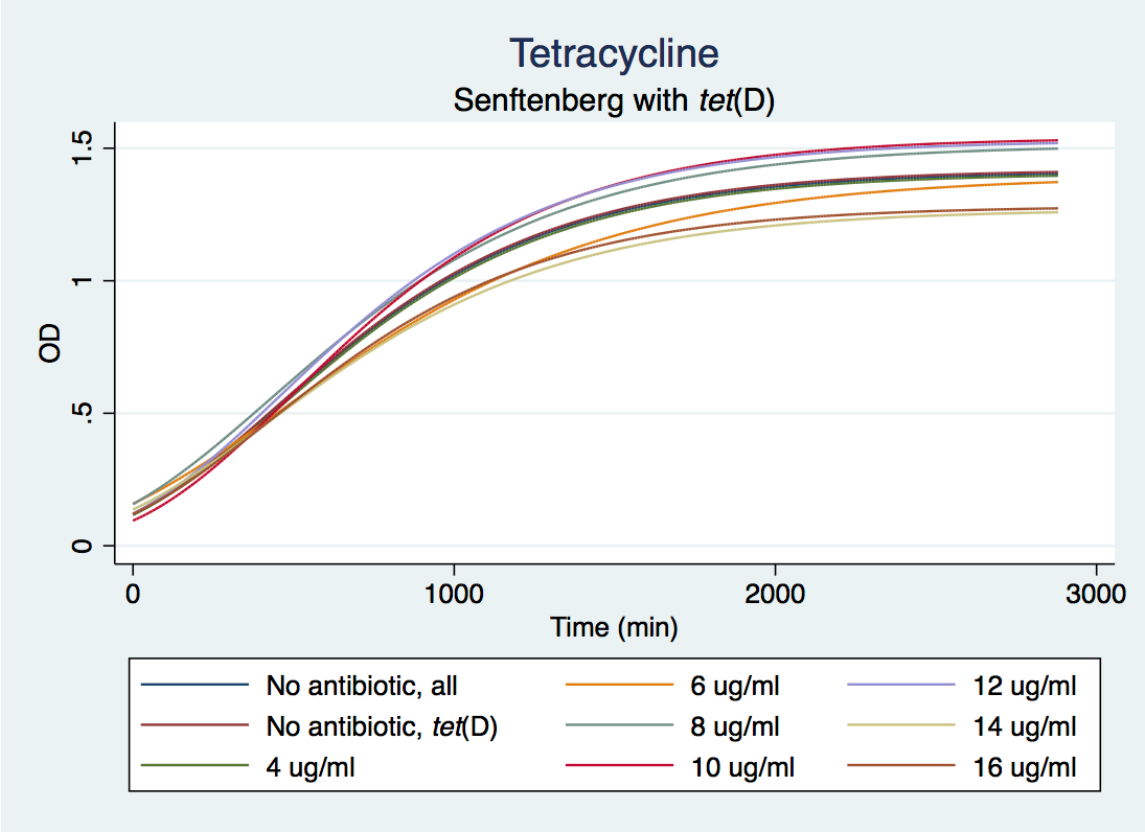


Figure 4-29 3-Parameter Gompertz fitted growth curves of *Salmonella* Senftenberg with *tet*(D) gene at different tetracycline concentrations

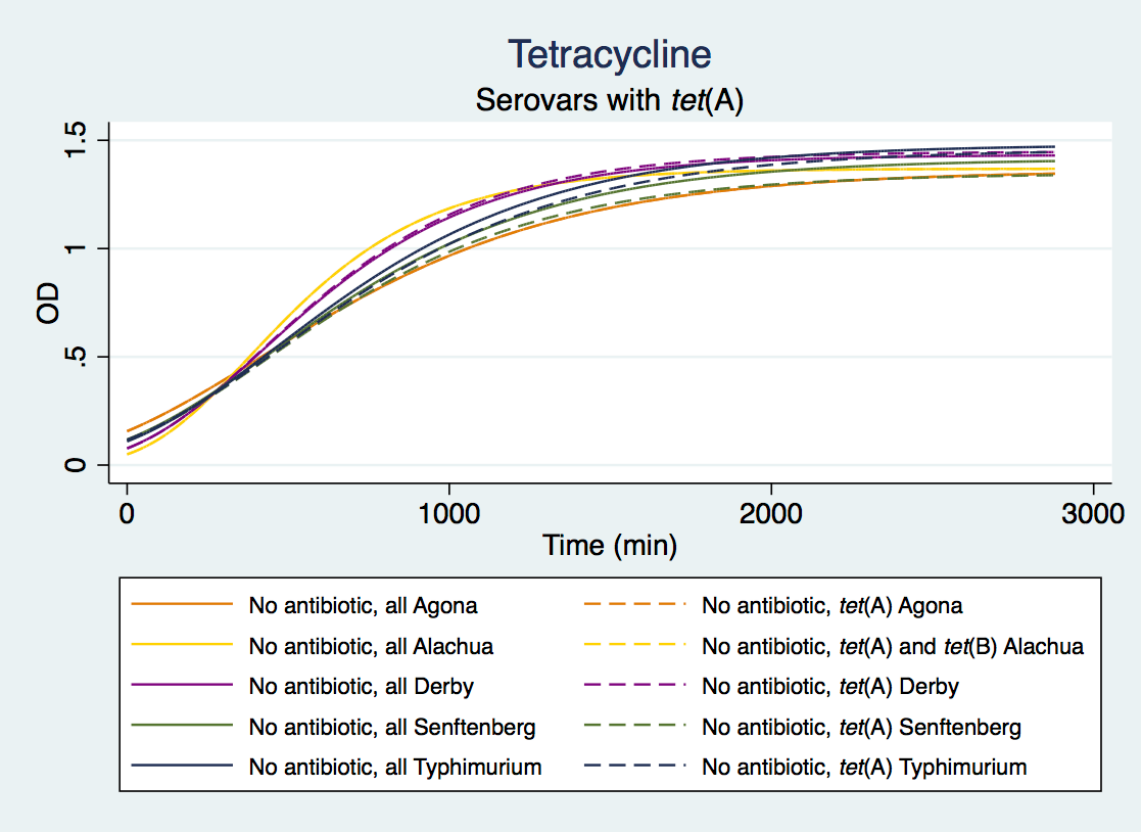


Figure 4-30 Fitness costs of *Salmonella* serovars with *tet*(A)

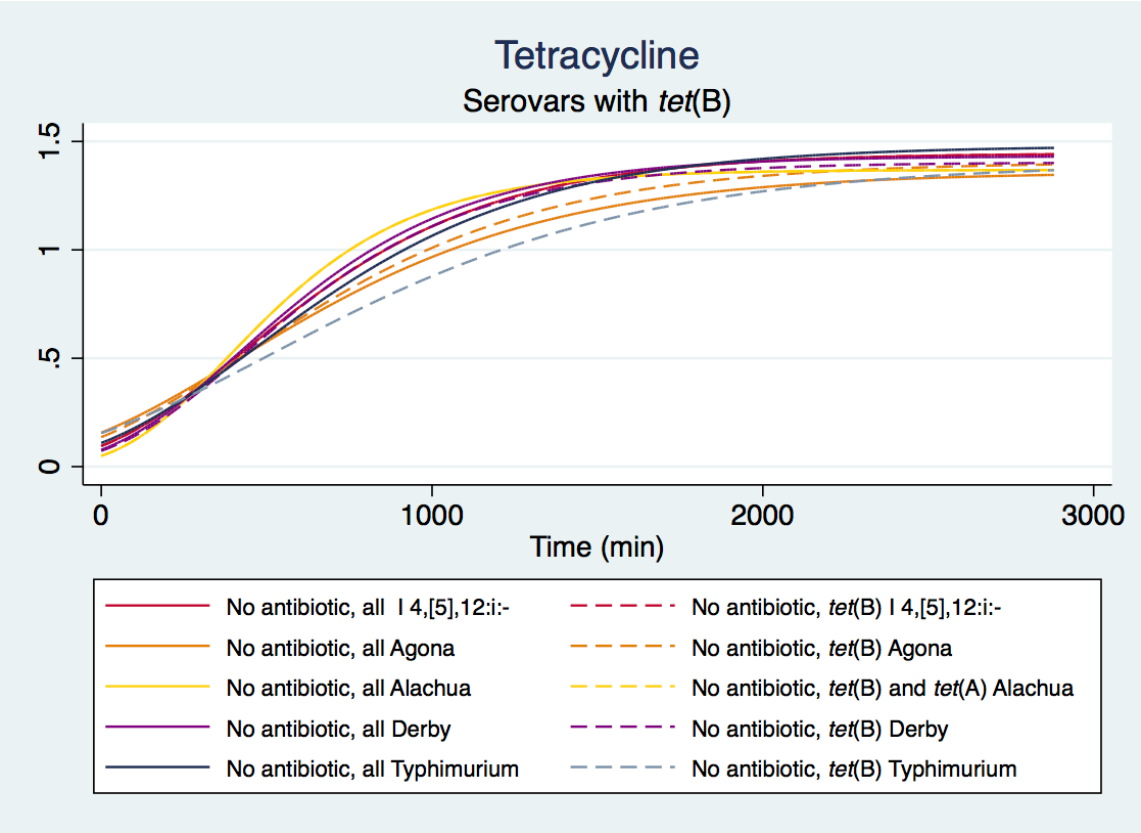


Figure 4-31 Fitness costs of *Salmonella* serovars with *tet*(B)

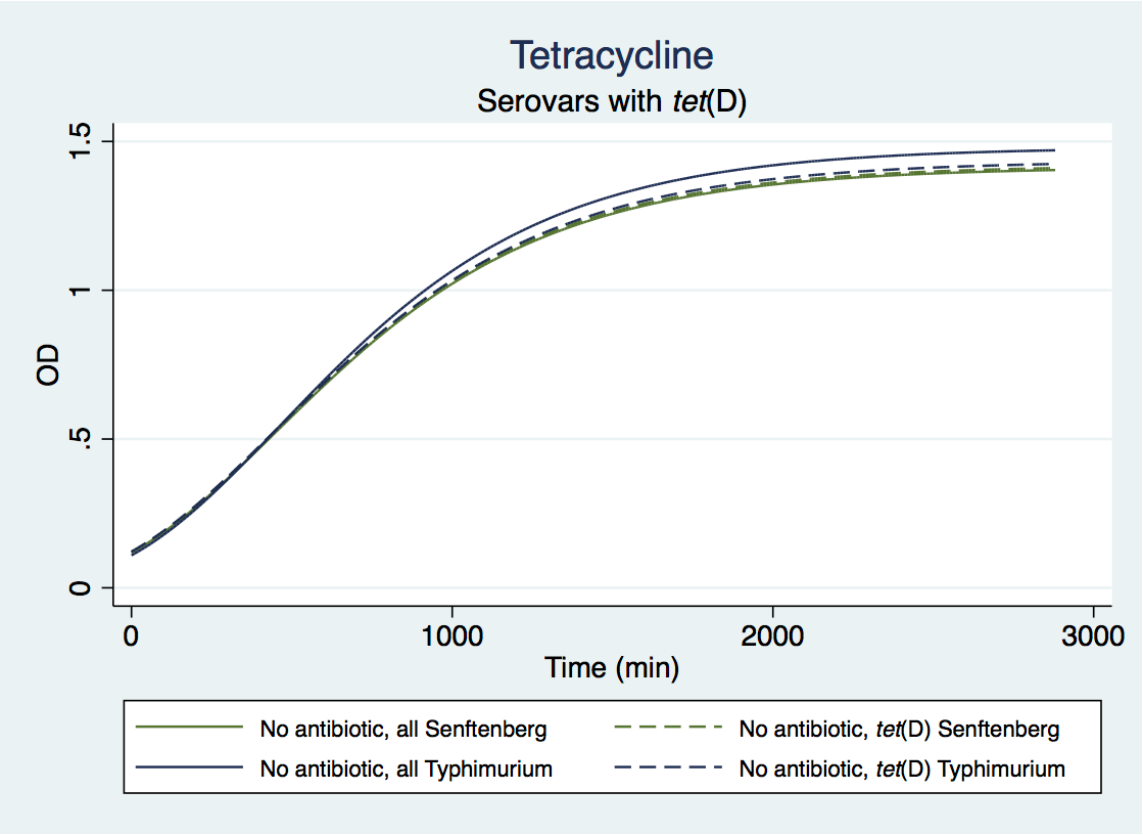


Figure 4-32 Fitness costs of *Salmonella* serovars with *tet*(D)

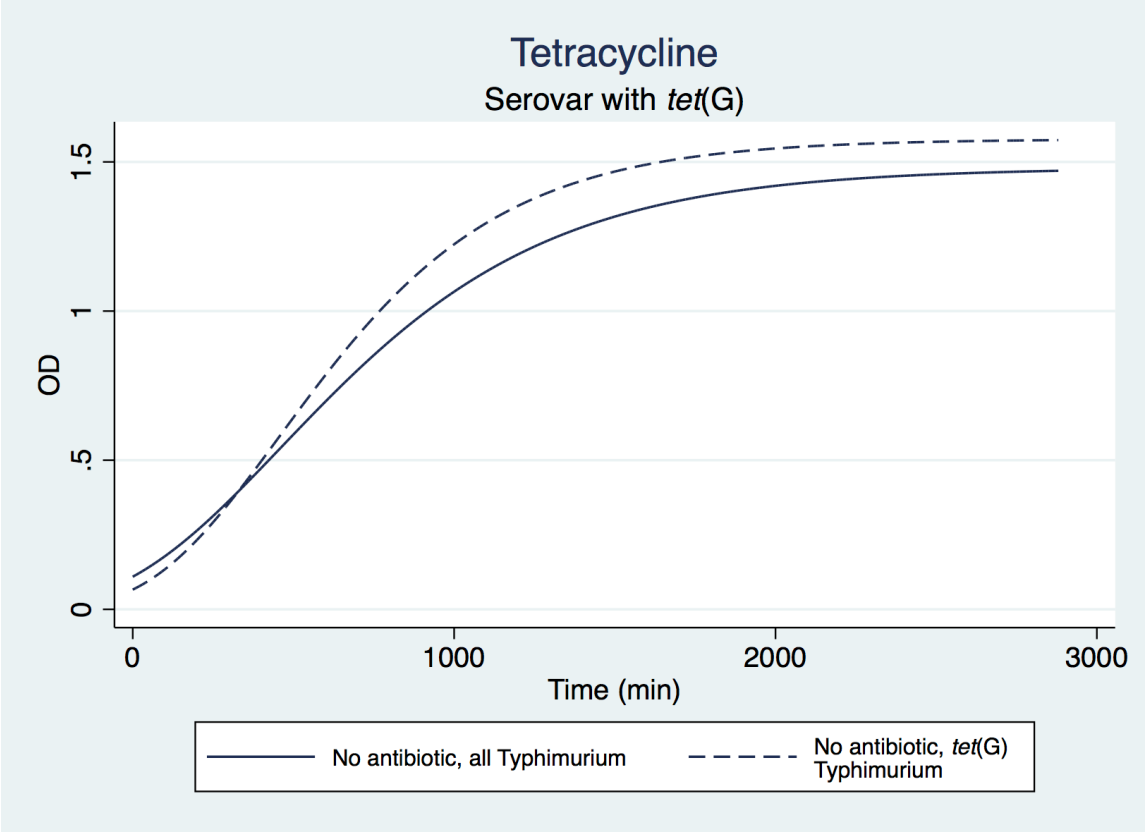


Figure 4-33 Fitness costs of *Salmonella* serovar with *tet*(G)

4.4. Swarm and swim ability

In vitro motility assays were performed on all *Salmonella* isolates (n = 125) to estimate and compare the swimming and swarming ability between the following *Salmonella* groups: biphasic *Salmonella* Typhimurium versus monophasic *Salmonella* I 4,[5],12:i:-, biphasic versus monophasic *Salmonella* strains overall, and multidrug resistant (resistant ≥ 3 antibiotic classes) versus non-multidrug resistance (resistant ≤ 2

antibiotic classes) strains. The Student's t-test was used to determine significant differences between the motility of these groups. Furthermore, a one-way ANOVA was used to determine significant differences of motility between two or more serovars. If there was a significant difference, the Bonferroni multiple comparison test was used to compare the motility of each serovar. A p-value less than 0.05 ($p \leq 0.05$) was statistically significant.

In Figure 4-34, a boxplot is shown to visualize the distribution of mean diameter values for each serovar in both swarm and swim assays. A brief comparison shows more variation among serovars in swimming than swarming motility at all time points (6, 12, and 18 h). Significant differences were observed between the swimming ability of biphasic (serovar Typhimurium) and monophasic (serovars I 4,[5],12:i:-, Derby, Senftenberg, Enteritidis, Montevideo, Agona, and Alachua) isolates at all time points: 6 ($p = 0.004$), 12 ($p = 0.019$), and 18 hours ($p = 0.023$) (Table 4-6). The swimming motility of biphasic *Salmonella* isolates was greater than monophasic *Salmonella* isolates. For swarming, there was only a significant difference at 6 hours ($p = 0.002$) where biphasic *Salmonella* isolates had a greater swarming ability than monophasic *Salmonella* isolates (Table 4-5). Similar results were observed between *Salmonella* I 4,[5],12:i:-, a monophasic strain, and *Salmonella* Typhimurium, a biphasic strain with no significant differences between these serovars in the swarming ability (Table 4-5). However, there were significant differences in swimming between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium at all time

points, where the swimming ability of *Salmonella* Typhimurium was greater than *Salmonella* I 4,[5],12:i:- (Table 4-6). The swimming and swarming ability was assessed for all MDR and non-MDR serotypes. Results show there were no significant differences in swarming between MDR and non-MDR isolates (Table 4-5). On the other hand, there were significant differences in swimming between MDR and non-MDR *Salmonella* isolates at all time points, with MDR isolates having a greater swimming ability than non-MDR isolates. (Table 4-6).

A statistically significant difference between the means in the swarming ability was observed among the different serovars only at 6 hours as determined by a one-way ANOVA ($p \leq 0.05$). The Bonferroni multiple comparison test showed significant differences between *Salmonella* Agona and *Salmonella* I 4,[5],12:i:-, *Salmonella* Agona and *Salmonella* Typhimurium, and *Salmonella* Senftenberg and *Salmonella* Typhimurium. *Salmonella* Typhimurium had greater swarming ability than *Salmonella* Agona and *Salmonella* Senftenberg, while *Salmonella* Agona had a greater swarming ability than *Salmonella* I 4,[5],12:i:- (Table 4-7). As per the Student's t-test, there were no significant differences between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium in swarming.

For swimming, there was a statistically significant difference in the means in swimming motility between serovars at all time points as determined by a one-way ANOVA. The Bonferroni multiple comparison test revealed significant differences

between *Salmonella* I 4,[5],12:i:- and other serovars such as Agona, Derby, Enteritidis, Senftenberg, and Typhimurium. *Salmonella* Agona, Senftenberg, and Typhimurium had greater swimming ability than *Salmonella* I 4,[5],12:i:- at 6 hours and 12 hours (Table 4-8). *Salmonella* Senftenberg swimming was also greater than *Salmonella* I 4,[5],12:i:- at 18 hours. The other monophasic serovars showed significantly different swimming ability than *Salmonella* I 4,[5],12:i:-, such as *Salmonella* Derby and Enteritidis at 12 hours; both of which had a greater swimming ability than *Salmonella* I 4,[5],12:i:-. However, by 18 hours there are no significant differences between the swimming of *Salmonella* I 4,[5],12:i:- and *Salmonella* Derby or *Salmonella* Enteritidis. *Salmonella* Agona had a lesser swimming ability than *Salmonella* Alachua at 6 and 12 hours and *Salmonella* Senftenberg at all time points (Table 4-8). Additionally, *Salmonella* Alachua displayed lesser swimming ability than *Salmonella* Enteritidis and *Salmonella* Montevideo at 6 and 12 hours, though it was not significant at 6 hours ($p = 0.062$). At both 6 and 12 hours, *Salmonella* serovars Derby, Enteritidis, and Monteviedo had a greater swimming ability than *Salmonella* Alachua; however, by 18 hours there were no significant differences. *Salmonella* Senftenberg was the only serovar with significant differences in swimming ability across all time points with greater swimming motility than all the other monophasic serovars, except *Salmonella* Alachua where it was not significant and greater until 18 hours (Table 4-8). However, *Salmonella* Senftenberg had a lesser swimming ability than biphasic *Salmonella* Typhimurium at all time points.

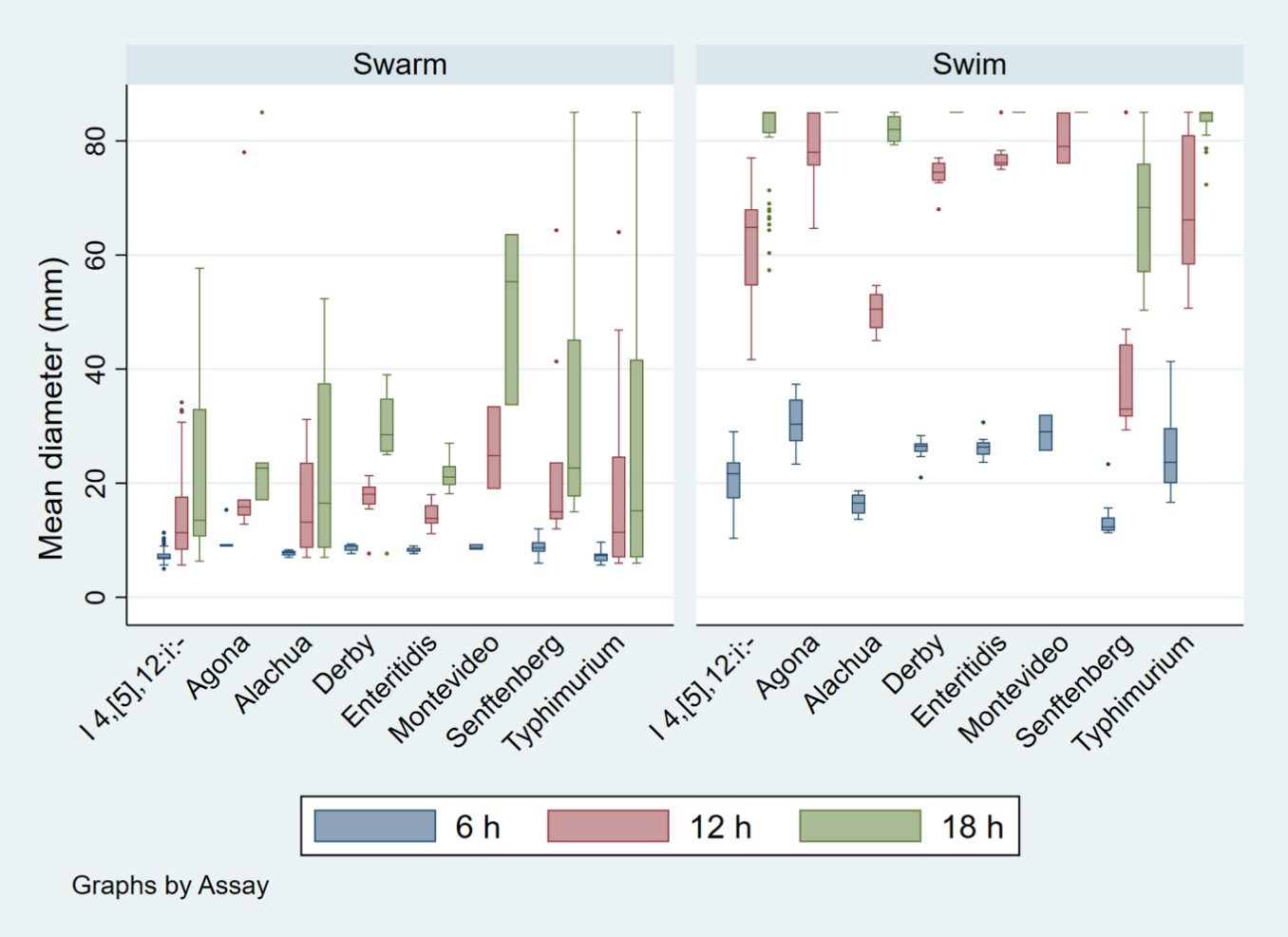


Figure 4-34 Boxplot visualization of motility ability across serovars

Table 4-6 Analysis of the mean diameter of the swarming ability of *Salmonella* groups compared in this study using the Student's t-test

SWARM ASSAYS *Student's t-test*					
Group comparison			6 h P >|t|	12 h P >|t|	18 h P >|t|
I 4,[5],12:i:-	vs	Typhimurium[†]	0.272	0.263	0.236
biphasic	vs	monophasic[‡]	0.002	0.784	0.560
MDR	vs	non-MDR[§]	0.858	0.328	0.288

[†]Comparing swarming ability of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates
[‡]Comparing swarming ability of biphasic and monophasic *Salmonella* isolates
[§]Comparing swarming ability of multi-drug resistant (MDR) and non-MDR *Salmonella* isolates

Table 4-7 Analysis of the mean diameter of the swimming ability of *Salmonella* groups compared in this study using the Student's t-test

SWIM ASSAYS *Student's t-test*					
Group comparison			6 h P >|t|	12 h P >|t|	18 h P >|t|
I 4,[5],12:i:-	vs	Typhimurium[†]	0.001	0.001	0.026
biphasic	vs	monophasic[‡]	0.004	0.019	0.023
MDR	vs	non-MDR[§]	0.000	0.000	0.020

[†] Comparing swimming ability of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates
[‡] Comparing swimming ability of biphasic and monophasic *Salmonella* isolates
[§] Comparing swimming ability of multi-drug resistant (MDR) and non-MDR *Salmonella* isolates

Table 4-8 Statistically significant comparisons of swarming ability between *Salmonella* serovars at 6 hours

SWARM ASSAYS *Multiple comparisons test, Bonferroni*			
Serotype comparison			6 h P >|t|
Agona	vs	I 4,[5],12:i:-	0.001
Typhimurium	vs	Agona	0.000
Typhimurium	vs	Senftenberg	0.019

Table 4-9 Statistically significant comparisons of swimming ability between *Salmonella* serovars at 6, 12, and 18 hours.

SWIM ASSAYS *Multiple comparisons test, Bonferroni*					
Serotype comparison			6 h P >|t|	12 h P >|t|	18 h P >|t|
Agona	vs	I 4,[5],12:i:-	0.000	0.002	1.0
Derby	vs	I 4,[5],12:i:-	0.541	0.038	1.0
Enteritidis	vs	I 4,[5],12:i:-	0.269	0.002	1.0
Senftenberg	vs	I 4,[5],12:i:-	0.001	0.000	0.000
Typhimurium	vs	I 4,[5],12:i:-	0.005	0.014	0.639
Alachua	vs	Agona	0.001	0.001	1.0
Senftenberg	vs	Agona	0.000	0.000	0.000
Derby	vs	Alachua	0.101	0.010	1.0
Enteritidis	vs	Alachua	0.061	0.001	1.0
Montevideo	vs	Alachua	0.062	0.010	1.0
Senftenberg	vs	Alachua	1.0	1.0	0.005
Typhimurium	vs	Alachua	0.032	0.027	1.0
Senftenberg	vs	Derby	0.000	0.000	0.000
Senftenberg	vs	Enteritidis	0.000	0.000	0.000
Senftenberg	vs	Montevideo	0.000	0.000	0.001
Typhimurium	vs	Senftenberg	0.000	0.000	0.000

4.5. Biofilm production and biofilm-related genes

Biofilm production of *Salmonella* was assessed using the 96-well microtiter dish biofilm formation assay (Figure 4-35) and classified into no, weak, moderate, and strong biofilm producer based on the OD_{600} values (Figure 4-36). Biofilm production varied across serovars; however most were weak biofilm producers (Figure 4-36). Specifically, 89% of *Salmonella* I 4,[5],12:i:- and about 95% of *Salmonella* Typhimurium isolates were weak biofilm producers. A few of the *Salmonella* I 4,[5],12:i:- isolates (~11%) did not form any biofilm (no biofilm producer), whereas only one *Salmonella* Typhimurium was a moderate biofilm producer. *Salmonella* serovars Agona and Alachua were weak biofilm producers. The majority of *Salmonella* Derby were weak biofilm producers (77%), while two were moderate biofilm producers. About 50% of *Salmonella* Enteritidis isolates were moderate biofilm producers while the other half were strong biofilm producers. *Salmonella* Montevideo was the only other serovar with strong biofilm production (~67%) but had one weak biofilm producer.

The Mann-Whitney and Kruskal-Wallis tests were used to determine the differences in biofilm formation between biphasic and monophasic strains and between *Salmonella* serovars. Significant differences were determined when the p-value ≤ 0.05. The Mann-Whitney test indicated there were significant differences in biofilm production between biphasic and monophasic *Salmonella* serovars. Moreover, the Kruskal-Wallis tests revealed there were statistically significant differences between two or more serovars.

Individual Mann-Whitney tests showed significant differences in biofilm formation of *Salmonella* Enteritidis in comparison to all other serovars (I 4,[5],12:i:-, Agona, Alachua, Derby, Senftenberg, and Typhimurium) except Montevideo (Table 4-8). There were also significant differences between *Salmonella* Montevideo and *Salmonella* serovars I 4,[5],12:i:-, Agona, Alachua, and Senftenberg. Finally, significant differences were also observed between *Salmonella* Typhimurium and *Salmonella* serovars Derby and Senftenberg.

Among the biofilm-related genes identified (*adrA*, *fimH*, *csg*A, *csgB*, *csgD*, *sirA*, *glyA*, *ompR*, *sdiA*, *sipB*, *sipC*, *luxS*, *pfs*, and *gcpA*), all serovars showed the presence of *adrA, csg*A, *csgB*, *csgD*, *fimH, sipB, sipC,* and *sirA* genes. None of the *Salmonella* serovars contained the *gcpA, glyA,* and *pfs* genes. However, three genes were detected differentially across *Salmonella* serovars: *luxS, ompR,*and *sdiA.* The *luxS* gene was found in all serovars and only absent in two *Salmonella* Typhimurium isolates. Similarly, the *ompR* gene was found in all serovars and only absent in one *Salmonella* Derby isolate. The presence of the *sdiA* gene varied between biphasic and monophasic *Salmonella* isolates. *Salmonella* Typhimurium was the only serovar that had either presence (~24%) or absence (~76%) of the *sdiA* gene among the isolates. *Salmonella* Derby, Enteritidis, and Montevideo showed presence of the *sdiA* gene in all the isolates, while *Salmonella* Agona, Alachua, I 4,[5],12:i:-, and Senftenberg isolates lacked the *sdiA* gene.

A 3-way linear mixed effects regression model was used to assess the effects of biofilm-related genes and phase characteristic by *Salmonella* serovars on biofilm production. Phase characteristic (monophasic and biphasic), serovar, and biofilm-related genes (*luxS, ompR,*and *sdiA*) were included as fixed effects and biological replicates was included as a random effect and nested into id (*Salmonella* isolates). Thus, id was treated as a random effect which signifies a random sample from a larger population to model the between-id variability. Furthermore, the response variable (OD, optical density) was natural log transformed. The intercept-only model with random effects showed nesting variances and that the biological replicates were not normally distributed. This signifies those biological replicates must be accounted for in the variability of the model. Each individual gene was run with the model. It was revealed that *sdiA* had a significant effect on biofilm formation, observed by an increase in ln(OD). The genes *ompR* and *luxS* had no effect on biofilm formation.

The final model included the phase characteristic and serovars to determine the effect *sdiA* had on biofilm formation of monophasic and biphasic strains as well as each of the individual *Salmonella* serovars. The marginal means were estimated and graphed by serovar with 95% confidence intervals (95%CI) (Figure 4-37). This showed that the presence of the *sdiA* gene had a significant positive effect on biofilm formation for *Salmonella* serovars Derby (-2.463524, -2.566904 to -2.360144), Enteritidis (95%CI, -1.620821 to -1.401519), Montevideo (-1.51117, -1.692276 to -1.334157), and

Typhimurium (-2.471328, -2.569403 to -2.373254). On the other hand, the absence of *sdiA* had a negative effect for *Salmonella* serovars I 4,[5],12:i:- (-2.693276, -2.739004 to -2.647548), Agona (-2.519319, -2.636541 to -2.402097), Alachua (-2.590674, -2.745745 to -2.435604), Senftenberg (-2.539543, -2.637618 to 2.441468), and Typhimurium (-2.634388, -2.692999 to -2.575777). As seen for the individual Mann-Whitney tests, *Salmonella* Enteritidis (*sdiA* = 1) had greater biofilm production than most *Salmonella* serovars without *sdiA*. *Salmonella* Montevideo (*sdiA* = 1) also had greater biofilm formation than serovars without *sdiA* (I 4,[5],12:i:-, Agona, Alachua, and Senftenberg). In contrast to *Salmonella* Enteritidis, Montevideo and Typhimurium with the *sdiA* gene, the presence of the *sdiA* gene, only had a small positive effect on biofilm formation on *Salmonella* Derby as only two were moderate biofilm producers and the rest were weak biofilm producers. Upon a closer evaluation of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium (Figure 4-38), the absence of *sdiA* had a negative impact on biofilm production as seen in monophasic *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates without the gene. Although the presence of *sdiA* had a positive effect on biofilm production in biphasic Typhimurium isolates.

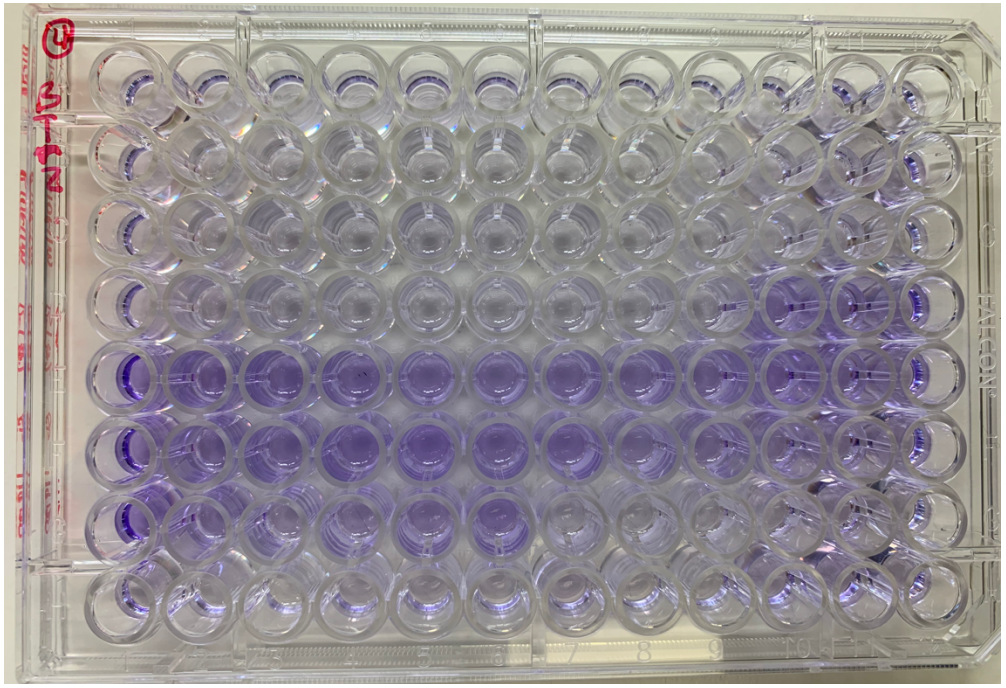


Figure 4-35 Biofilm formation in the 96-well microtiter dish

Table 4-10 Statistically significant comparisons of biofilm production between *Salmonella* serovars

BIOFILM PRODUCTION *Wilcoxon-Mann-Whitney rank-sum test*			
Serovar comparison			P >|z|
Enteritidis	vs	I 4,[5],12:i:-	0.000
Enteritidis	vs	Agona	0.001
Enteritidis	vs	Alachua	0.004
Enteritidis	vs	Derby	0.014
Enteritidis	vs	Senftenberg	0.001
Enteritidis	vs	Typhimurium	0.000
Montevideo	vs	I 4,[5],12:i:-	0.000
Montevideo	vs	Agona	0.003
Montevideo	vs	Alachua	0.018
Montevideo	vs	Senftenberg	0.010
Typhimurium	vs	Derby	0.033
Typhimurium	vs	Senftenberg	0.000

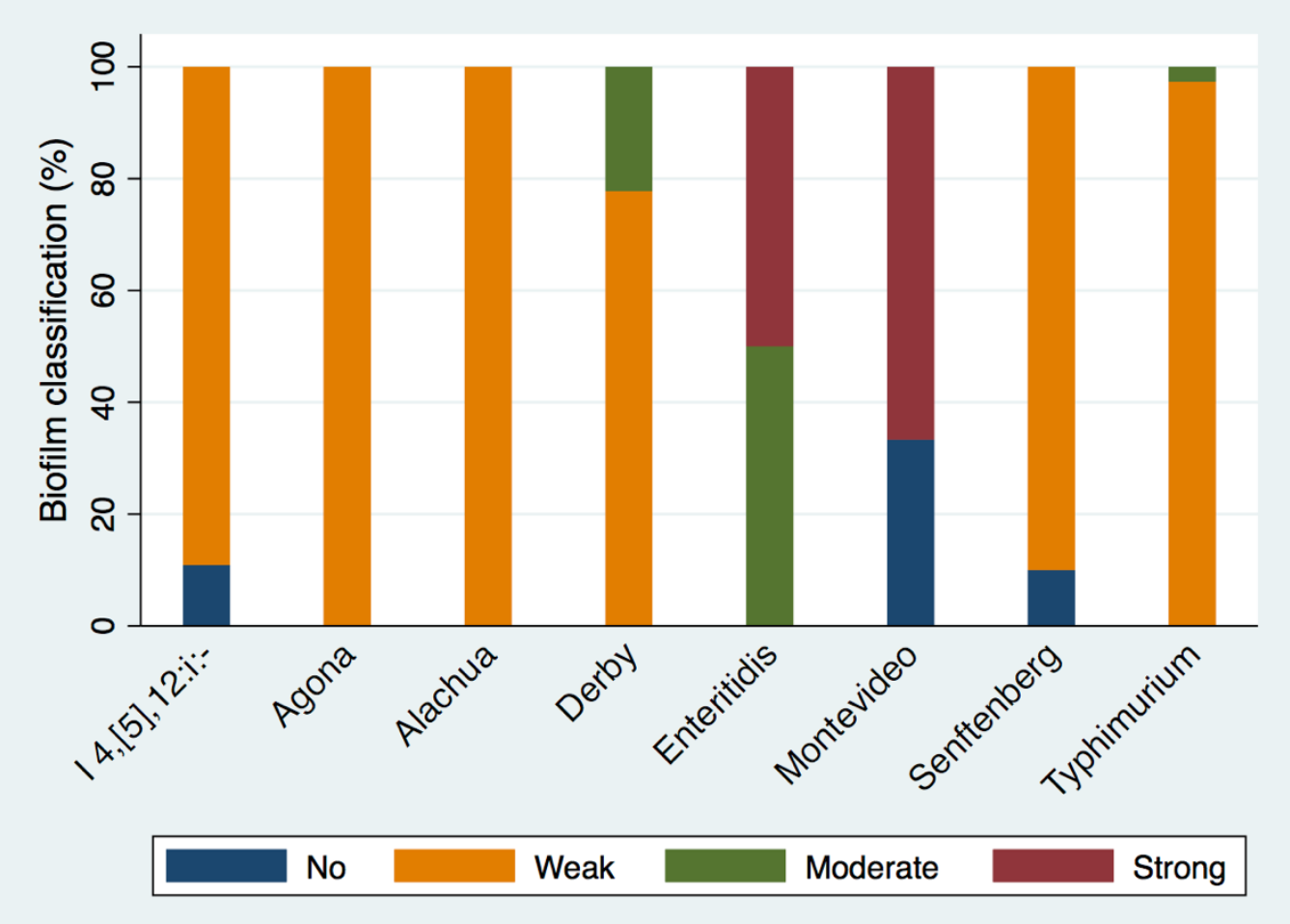


Figure 4-36 Biofilm production classification of *Salmonella* isolates

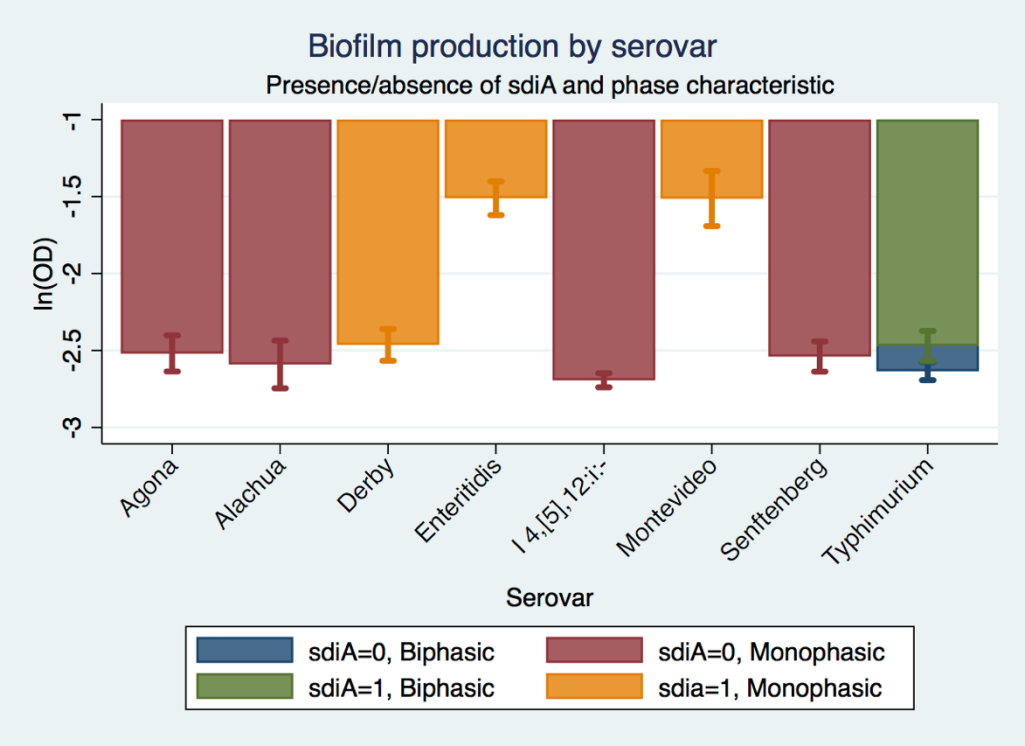


Figure 4-37 Marginal mean estimates of ln(OD) and the 95% confidence intervals, biofilm production by serovar

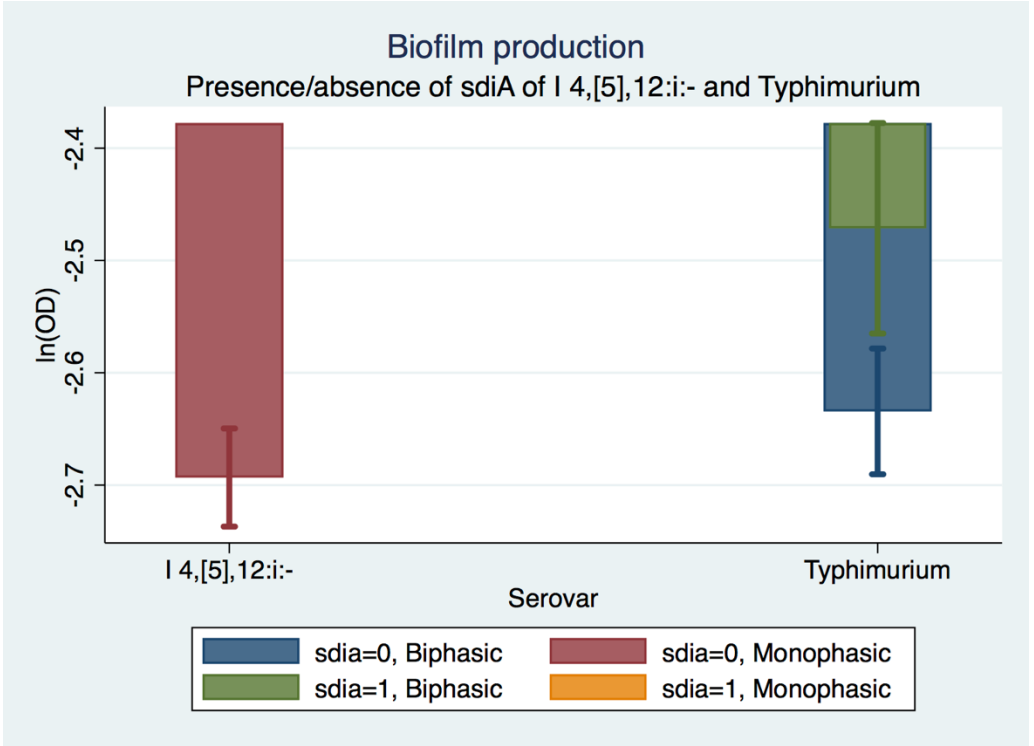


Figure 4-38 Marginal mean estimates of ln(OD) and the 95% confidence intervals, biofilm production of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium

4.6. Presence of HMT genes in Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

The heavy metal tolerance (HMT) genes for copper (*pcoABCDRS*), silver (*silABCEFPS*) and mercury (*merACDEPRT*) were identified in *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium for comparison (Figure 4-39). Both *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium showed presence of chromosomal copper resistance genes (*bhsA, comR, copA, cuePR, cutACER*) across all isolates. However, all 46 *Salmonella* I 4,[5],12:i:- isolates harbored all the plasmid-borne copper resistance genes (*pcoABCDRS*), whereas six *Salmonella* Typhimurium (~15.8%) harbored *pcoABCDR* and four (10.5%) only harbored *pcoS*. Furthermore, all 46 *Salmonella* I 4,[5],12:i:- isolates also harbored mercury resistance genes, *merACDEPT* and *merR1* (Figure 4-40). Only three *Salmonella* I 4,[5],12:i:- isolates (~6.5%) contained the *merR* gene. In contrast, four *Salmonella* Typhimurium isolates (~10.5%) harbored *merACDEPRT*, seven (~18.4%) *merADEFPT* and *mer2*, six (~15.8%) only had *mer*R2 and *merT*. The remaining *Salmonella* Typhimurium isolates, (n = 21, 55%) contained no mercury resistance genes. All *Salmonella* I 4,[5],12:i:- isolates also contained *silABCEFPS,* while only 6 *Salmonella* Typhimurium (~15.8) harbored *silABCEFPS* (Figure 4-41). All *Salmonella* I 4,[5],12:i:- isolates in our study contain the same HMT gene profile (*pcoABCDRS, silABCEFPS,* and *merACDEPT* and *merR1*). Three *Salmonella* I 4,[5],12:i:- isolates also harbored *merR*. There was more variation in the HMT gene profile of *Salmonella* Typhimurium isolates.

Six *Salmonella* Typhimurium isolates contained *pcoABCDRS, silABCEFPS,* and *merADEPT* and *merR2*. The four *Salmonella* Typhimurium isolates that harbored *merACDEPRT* also had the *pcoS* gene. One *Salmonella* Typhimurium contained only mercury tolerance genes *merADEPT* and *merR2*, and six had only the *merR2* and *mer1* mercury genes. Twenty-one *Salmonella* Typhimurium isolates only contained the *bhsA, comR, copA, cuePR, cutACER* HMR genes.

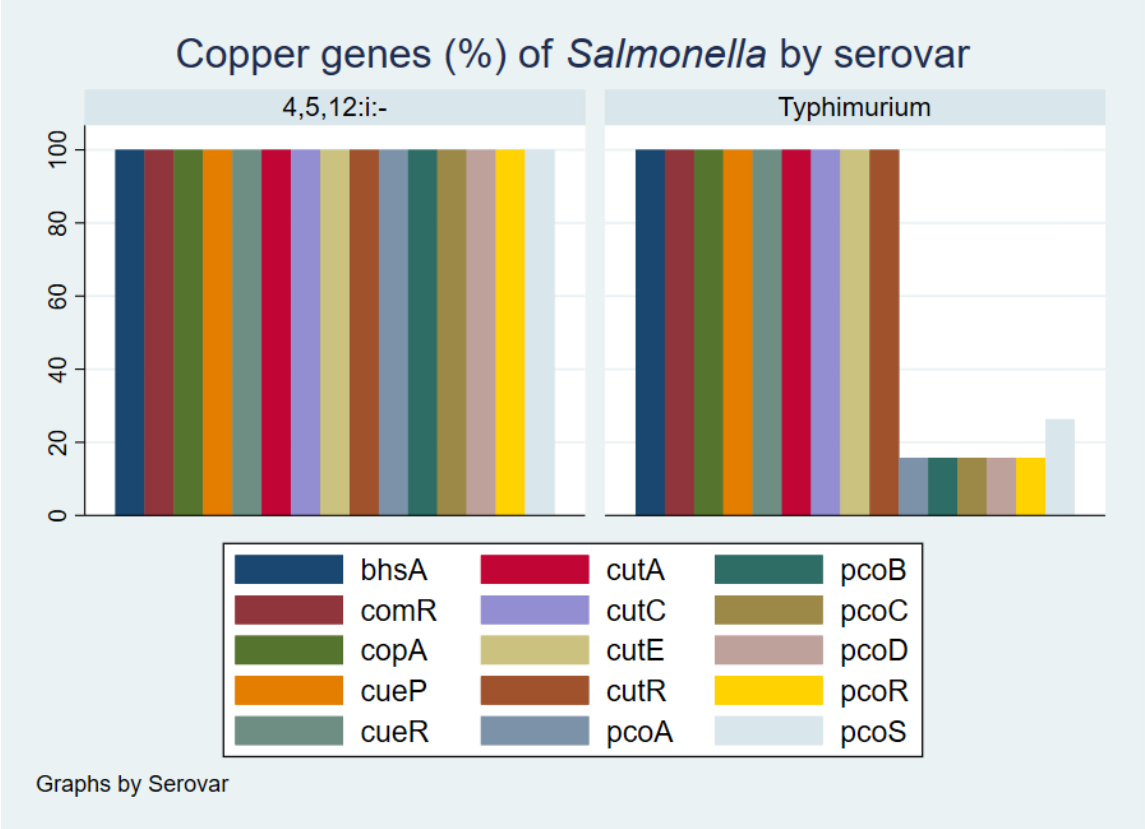


Figure 4-39 Comparison of copper tolerance genes present between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates

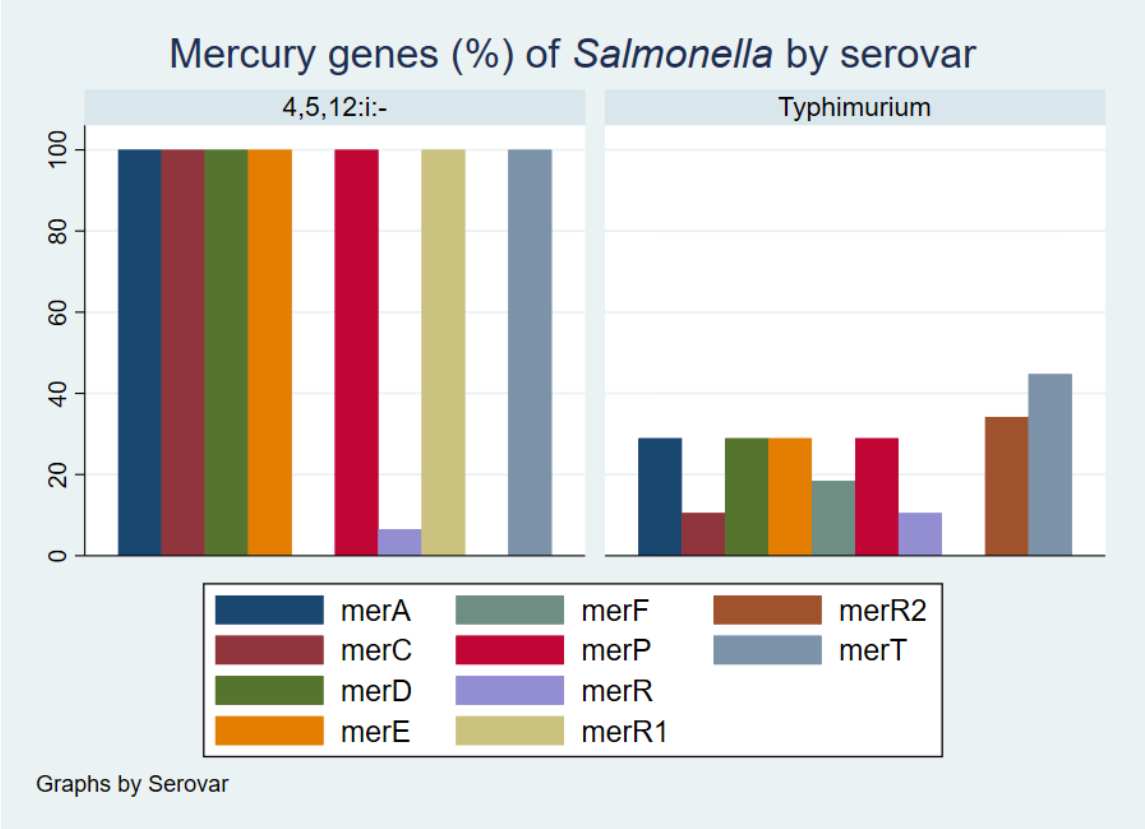


Figure 4-40 Comparison of mercury tolerance genes present between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates

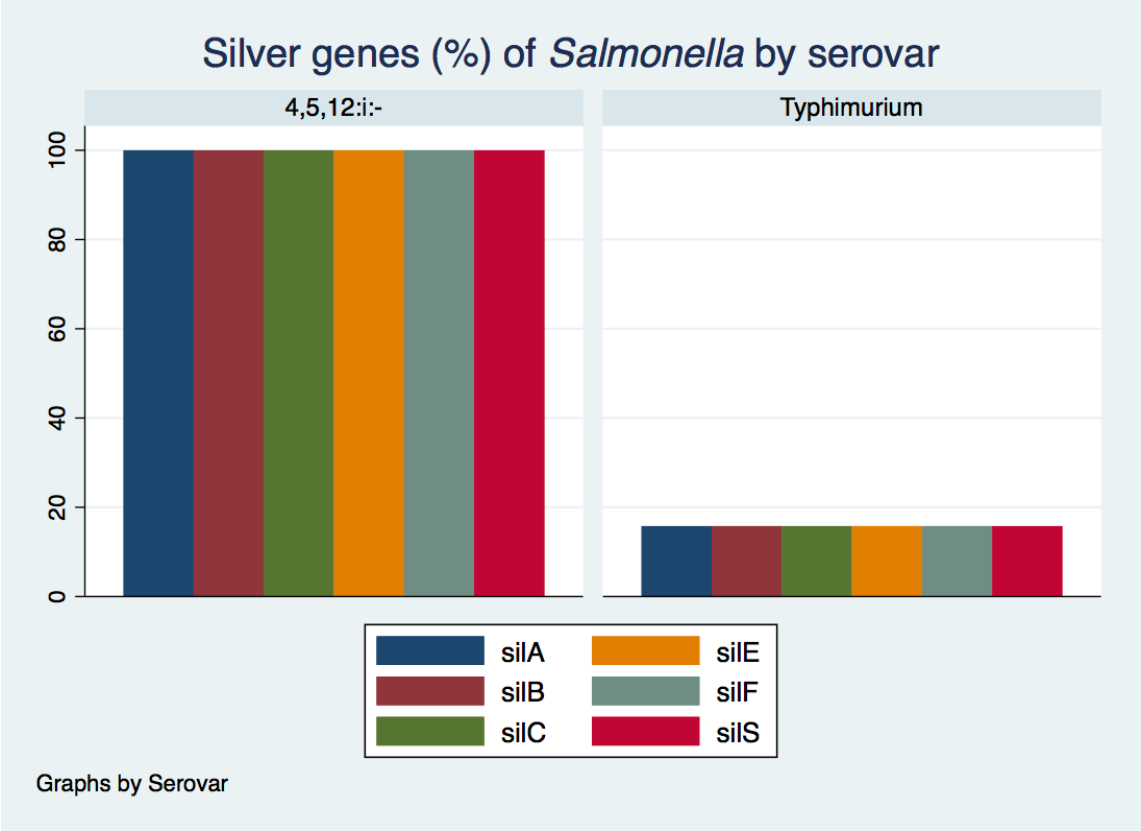


Figure 4-41 Comparison of silver tolerance genes present between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates

4.7. Annotation and mapping of **Salmonella** *I 4,[5],12:i:-*

All *Salmonella* I 4,[5],12:i:- isolates (n = 46) in our study were mapped to the *Salmonella* Typhimurium str. LT2 reference genome (NC_003197) to identify deletions and/or mutations in the phase 2 flagella locus region via Geneious Prime v2019.2.1. The genes of interest in this region were *flj*B (FljB; phase 2 flagellar antigen), *flj*A (FliC repressor), *hin* (Hin recombinase), and *iroB* (iron-related glycosyltransferase). When mapped to the *Salmonella* Typhimurium reference genome, a deletion of the phase 2 flagellar antigen region is observed in all *Salmonella* I 4,[5],12:i:- isolates in our study (e.g. Figure 4-42). The *Salmonella* I 4,[5],12:i:- deletion is approximately 15 kb when compared to *Salmonella* Typhimurium. As seen in Figure 4-42, the following genes were observed in the deletion: *flj*B, *flj*A, and *hin*. However, the *iroB* gene remained conserved in *Salmonella* I 4,[5],12:i:- isolates. Additional deletions included putative transposases, integrases, and inner membrane protein, and hypothetical proteins.

Furthermore, SnapGene Viewer v5.2.4 was used to detect and visualize any insertions of mobile genetic elements within the phase 2 flagellar antigen region of two hybrid assemblies (long and short reads) of *Salmonella* I 4,[5],12:i:- isolates. Figure 4-43 shows the phase 2 flagellar antigen region of isolate B70HEB11 which had the MDR genotype, *aadA1*, $bla_{\text{TEM-1}}$, *strA-strB*, *sul*1, *sul*2, *tet*(B) while isolate F70H3 had the same MDR genotype with additional AMR genes *aadA2*, *aph(3')-Ia*, $bla_{\text{SHV-12}}$, *dfrA19*, *mcr*-9, *qnrB*2, and *tet*(D). An insertion of an antimicrobial resistance and heavy metal tolerance

gene cassette surrounded by mobile genetic elements was detected within the *flj*B region in both *Salmonella* I 4,[5],12:i:- isolates. The insertion was approximately 28 kb in length; in both cases, the AMR genes encoding the ASSuT R-type ($bla_{\text{TEM-1}}$, *strA*-*strB*, *sul*2, *tet*(B)) were detected within this region along with the *merACDERT* mercury operon.

Figure 4-42 Flagellar phase 2 region of *Salmonella* I 4,[5],12:i:- mapped to *Salmonella* Typhimurium LT2 strain using Geneious

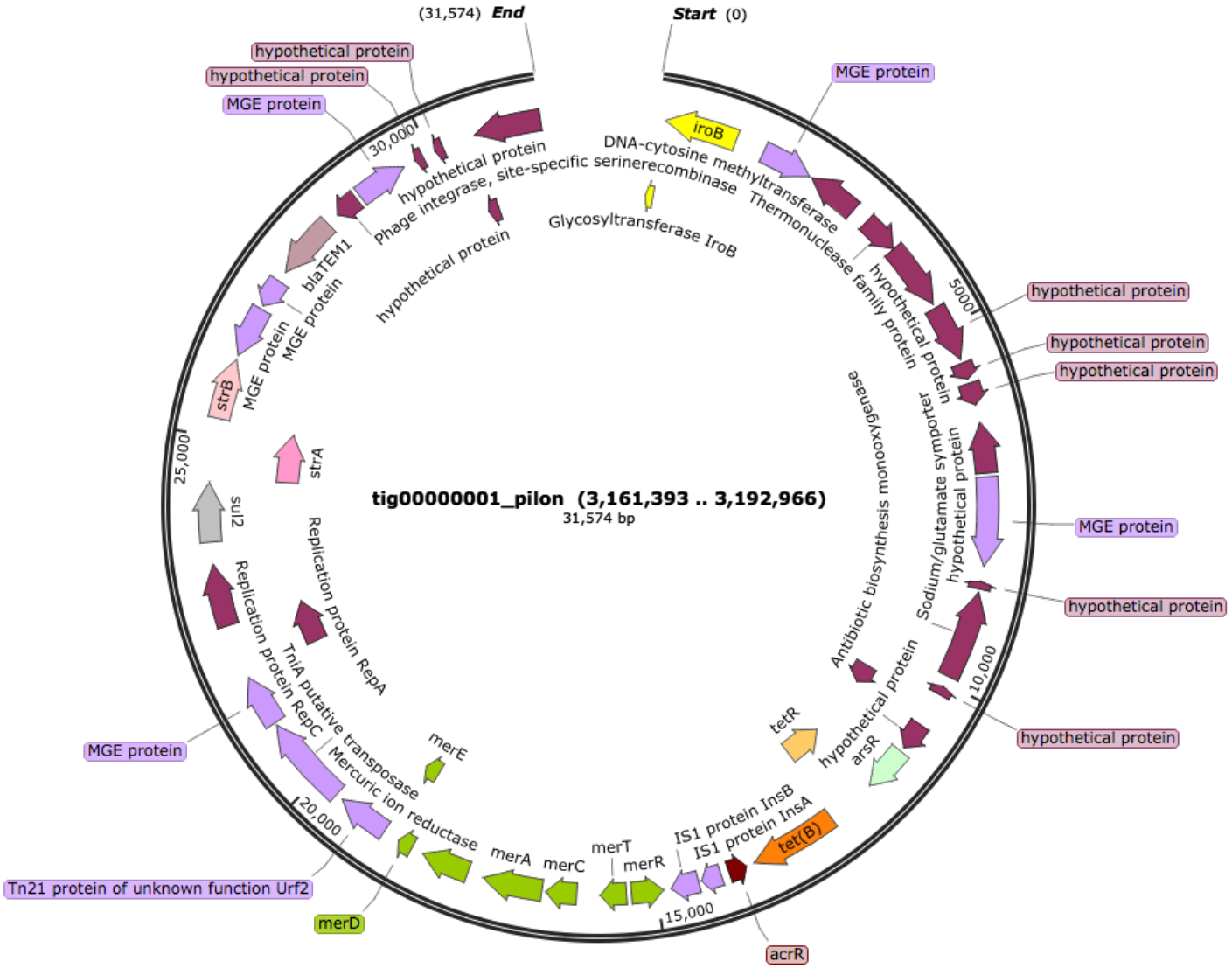


Figure 4-43 Flagellar phase 2 region of a *Salmonella* I 4,[5],12:i:- hybrid assembly visualized using SnapGene Viewer

4.8. Phylogenetic analyses of Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium strains*

The phylogenetic relationship of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes from our study were analyzed along with selected genomes that were publicly available on NCBI. Publicly available genomes were selected based on Hierarchical Clustering of Core-genome (HeirCC) MLST on Enterobase. Maximum-likelihood trees were created using IQ-TREE v1.6.12 using the reference genome *Salmonella* Enteritidis str. 18569 and visualized using Microreact with corresponding metadata. Nodes were labeled by sequence type of *Salmonella* strains and several subtrees were created to take a closer look at relationships between strains. The ASSuT phenotype data was obtained from the antimicrobial susceptibility testing results. Isolates showing the ASSuT genotype includes genes that give resistance to ampicillin, streptomycin, sulfonamides, and tetracycline as seen in Table 2-3. Isolates exhibited a MDR genotype if they had AMR genes encoding resistance to three or more antibiotic classes. Because the branches were not visible due to the outgroup reference genome, subtrees were created to enlarge certain clades and clusters of interest.

4.8.1. Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium genomes from our study*

Phylogenetic analyses were performed on our collection of isolates to determine if there was any relationship between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains isolated from swine. There were a total of 47 *Salmonella* I 4,[5],12:i:- and 38

Salmonella Typhimurium genomes from our study included in the analyses. One isolate previously classified as *Salmonella* Typhimurium was classified as *Salmonella* Infantitis by SISTR2 and SeqSero with GHRU; the misclassification of the serovar may have occurred when pulling several metadata files together. Therefore, from this moment on there are a total of 37 *Salmonella* Typhimurium isolates/genomes. Figure 4-44 shows a phylogenetic tree of *Salmonella* I 4,[5],12:i:- (n = 46) and *Salmonella* Typhimurium (n = 37) genomes with the reference genome *Salmonella* Enteritidis. It is important to note that we only included 37 *Salmonella* Typhimurium isolates representing the 299 Typhimurium isolates from the study conducted at ARS, USDA.

In Figure 4-45, you can see two distinct clades grouped by sequence type, one with ST19 *Salmonella* Typhimurium strains (Clade A) and a second with ST34 *Salmonella* I 4,[5],12:i:- (Clade B). In Clade A, ST19 *Salmonella* Typhimurium was isolated throughout the year 2015. There are a few closely related strains that were grouped together by months such as May, July, and November (Figure 4-46). On the other hand, the majority of ST34 *Salmonella* I 4,[5],12:i:- were isolated in September (~40%) and November (~36%); strains isolated in these months were closely related to each other as shown in Figure 4-47. Thus, more variation in months of isolation was seen in *Salmonella* Typhimurium than in *Salmonella* I 4,[5],12:i:- isolates. When looking at the sample type, both *Salmonella* Typhimurium and *Salmonella* I 4[5],12:i:- were isolated from cheek meat and head trim across different months. Most *Salmonella* Typhimurium isolates were from

cheek meat (~70%), while ~32% were from head trim. The case is opposite for *Salmonella* I 4,[5],12:i:-, where most were isolated from head trim (~62%) compared to cheek meat (~36%). There were groups of closely related *Salmonella* I 4,[5],12:i:- isolates from cheek meat and head trim. Furthermore, it was observed that the 39 of the 47 *Salmonella* I 4,[5],12:i:- were isolated using the enrichment culture method (Enriched), whereas only seven were isolated from EB Count Plates. More than half of the *Salmonella* Typhimurium (~59%) were isolated from EB Count while the rest (~41%) were isolated using the enrichment culture method. For the most part, closely related isolates further formed smaller clusters based on the selective media.

Furthermore, looking into the antimicrobial resistance phenotype and genotype of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains, a distinction was observed between the two serovars. Figure 4-46 takes a closer look at ST19 *Salmonella* Typhimurium in Clade A and shows two smaller clades formed (Clade A1 and Clade A2). In Clade A2, all the *Salmonella* Typhimurium were multidrug-resistant, showed phenotypic resistance to ASSuT, and harbored the $bla_{\text{CARB-2}}$, *floR*, *sul*1, and *tet*(G) resistance genes. Three isolates showed additional presence of *aad*A1 and *aad*A13 while seven harbored the $bla_{\text{TEM-1}}$ resistance gene, giving rise to the ASSuT genotype. Two of the three isolates with *aad*A genes were closely related to one other isolate with differences in the *aad*A gene, harboring *aad*A2 instead of *aad*A1 and *aad*A13. These closely related isolates were all isolated in the month of November. The seven isolates

harboring *bla*$_{\text{TEM-1}}$ were closely related to each other but were not distinctly isolated from a specific month or sample type. In Clade A2, there are two distinct groups of *Salmonella* Typhimurium. One group was multidrug-resistant (~46%), with five isolates showing phenotypic resistance to ASSuT, and all harboring the *strA-strB*, *sul*1, and *tet* (*tet*(A) or *tet*(D)) resistance genes. There were four closely related strains equally isolated from cheek meat and head trim harbored *tet*(A) as well as *bla*$_{\text{TEM-1.}}$ Three of them were isolated in July and one in May. These four isolates were closely related to two others isolated in September from cheek meat with *bla*$_{\text{TEM-1}}$. A different set of four closely related isolates were isolated in March and showed additional resistance genes such as *aac*(6')-IIc, *aph*(3')-Ia, *bla*$_{\text{TEM-1}}$, *dfr*A19, *ere*A, *mcr*-9, *qnr*B2, *sul*2, and *tet*(D) and showed the ASSuT phenotype. The second group was phenotypically pan-susceptible (n = 9) but harbored a single resistance gene (*bla*$_{\text{TEM-1}}$), and all were isolated from cheek meat in May.

Aforementioned, *Salmonella* I 4,[5],12:i:- were all multidrug-resistant showing the common ASSuT genotype found in *Salmonella* I 4,[5],12:i:- strains with the *bla*$_{\text{TEM-1}}$, *strA-strB, sul*2, and *tet*(B) genes and all but one showed the ASSuT phenotype. In Figure 4-47, Clade B can be seen to form two separate sub-clades, Clade B1 and Clade B2. In Clade B1 (n = 19) all *Salmonella* I 4,[5],12:i:- were isolated in September and additionally harbored the *qnrB*19 gene. There were some *Salmonella* I 4,[5],12:i:- isolates that also harbored *aad*A resistance genes such as *aad*A1, *aad*A2, and/or *aad*A13. However, there was no relationship with the presence of this gene and closely related isolates. One

exception was observed for three closely related *Salmonella* I 4,[5],12:i:- isolates that grouped together. All these three isolates harbored the *aad*A2 and were isolated from cheek meat. Similarly, other closely related strains were grouped by sample type in Clade B1 with the majority isolated from cheek meat (~32%) compared to head trim (~19%). Contrastingly, *Salmonella* I 4,[5],12:i:- isolates in Clade B2 (n = 27) were mainly isolated from head trim (~82%) and other sampling months: January (n = 1), March (n = 7), May (n = 3), and November (n = 17). There are two distinct clades of isolates where one clade (B2a) does not harbor *qnr*B19 and rarely harbor *aad*A, while the second clade (B2b) harbors *qnr*B19 and *aad*A genes. In the clade that does harbor *qnr*B19, there were isolates from every month and more isolated from head trim than cheek meat. Within this clade, a smaller cluster of three closely related strains is observed. These were isolated in November and harbored additional antimicrobial resistance genes like *aph*(3')-Ia, *dfrA*19, *mcr*-9, *qnr*B2, *sul*1, and *tet*(D). As in Clade B1 a similar clade of *Salmonella* I 4,[5],12:i:- isolates can be observed in Clade B2 where closely related strains (n = 14) are isolated only in the month of November, almost all harbored the *qnr*B19 gene, and some harbored *aad*A resistance genes. In this clade, only two isolates were isolated from cheek meat while the majority were from head trim.

Salmonella I 4,[5],12:i:- (Clade B) and *Salmonella* Typhimurium (Clade A) formed their own clade based on their antigenic profile and sequence type (Figure 4-45).

Overall, it can be observed that genetic relatedness within serovars was strongly based on the month of isolation and genotypic resistance profiles.

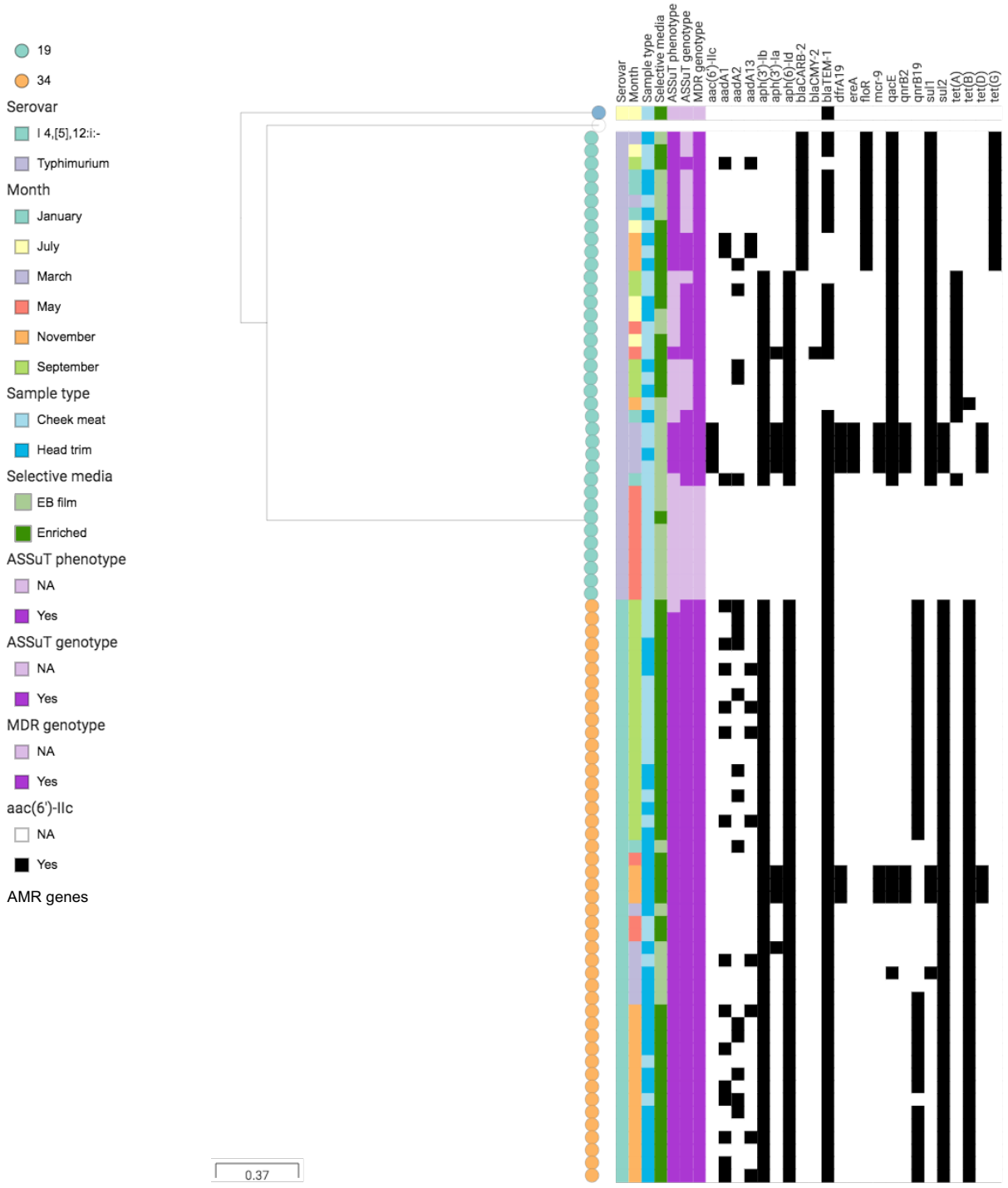


Figure 4-44 Phylogenetic tree of *Salmonella* I 4,[5],12:i:- (n = 46) and *Salmonella* Typhimurium (n = 37) from our study with *Salmonella* Enteritidis str. 18569 included as a reference strain

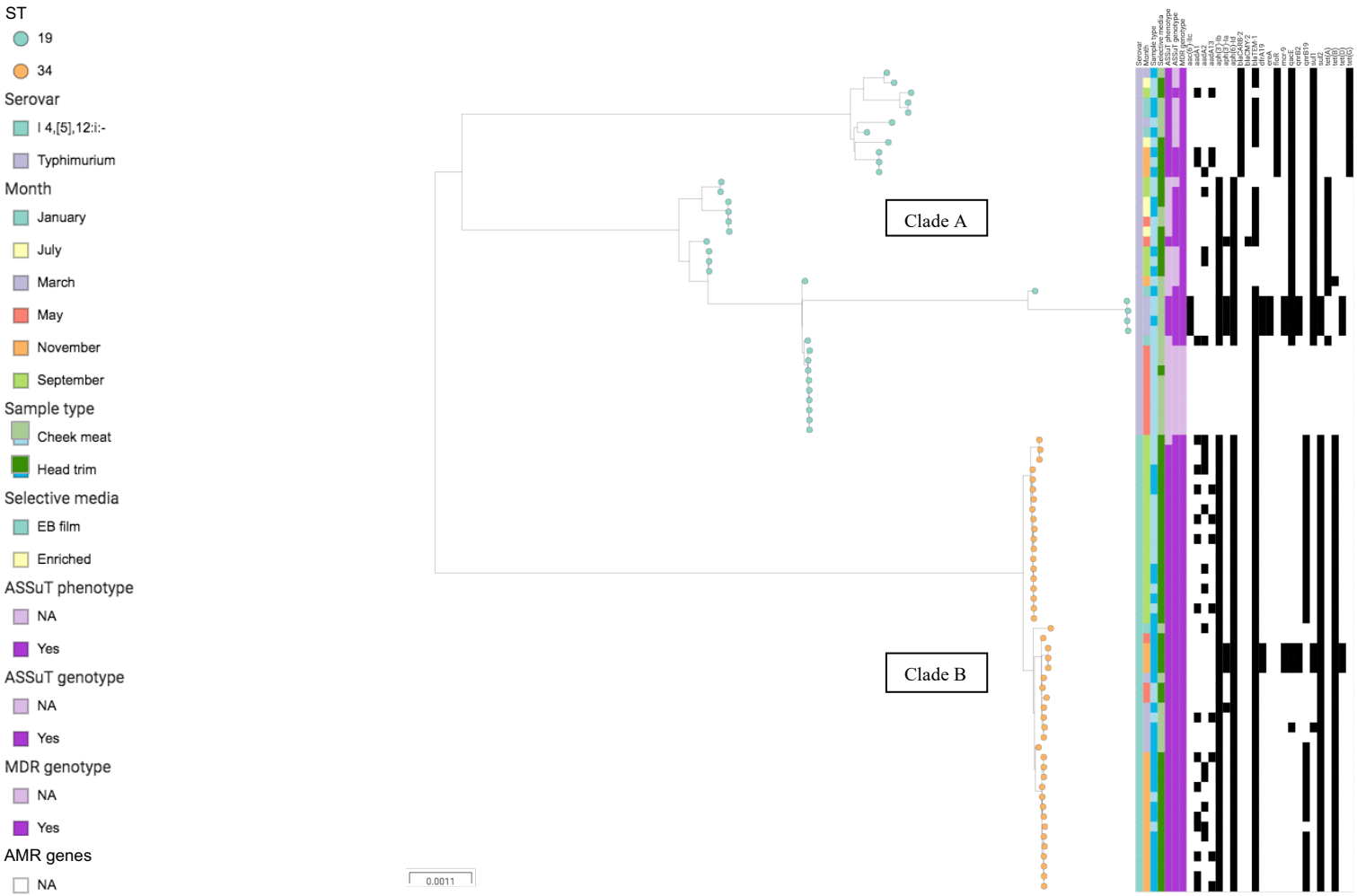


Figure 4-45 Subtree of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains from our study

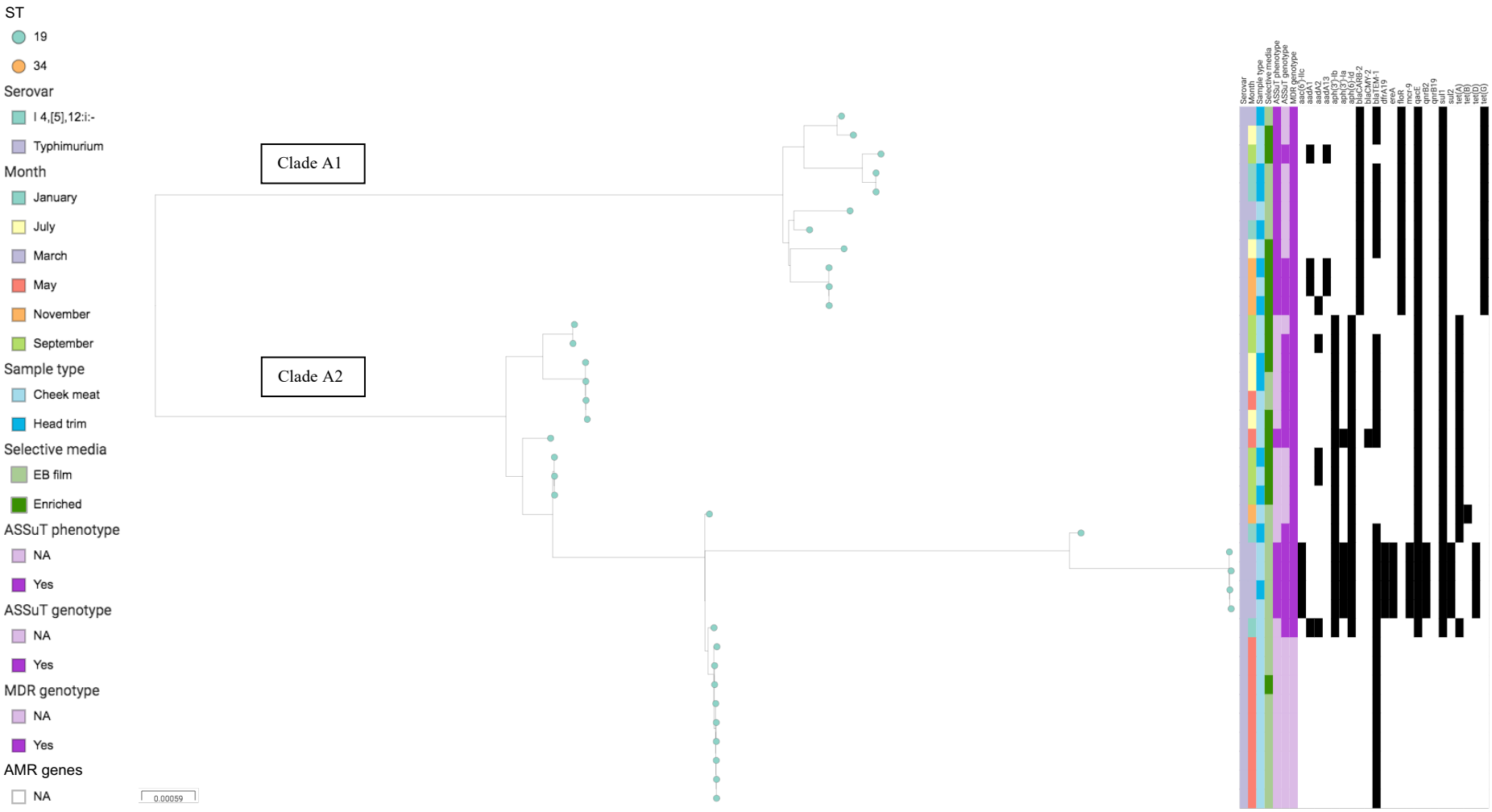


Figure 4-46 Clade A (from the subtree in Figure 4-45) representing the *Salmonella* Typhimurium (n = 38) from our study

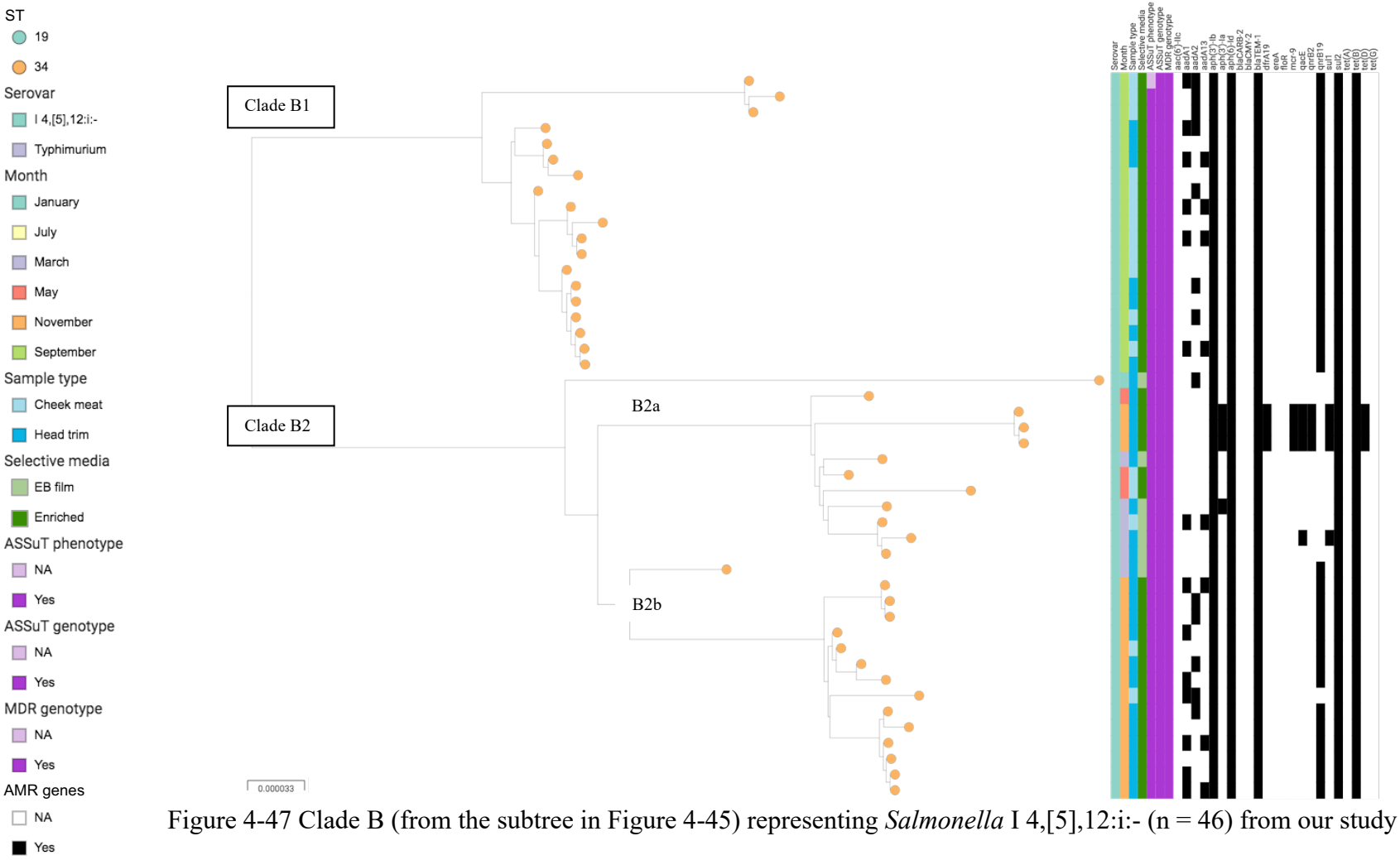


Figure 4-47 Clade B (from the subtree in Figure 4-45) representing *Salmonella* I 4,[5],12:i:- (n = 46) from our study

4.8.2. Salmonella *I 4,[5],12:i:- from our study and publicly available* Salmonella *I 4,[5],12:i:- genomes*

A second phylogenetic analysis was completed with our *Salmonella* I 4,[5],12:i:- genomes (n = 46) and selected publicly available *Salmonella* I 4,[5],12:i:- genomes (n = 655) to determine if there was any relationship based on deletion profile of the phase 2 flagellar antigen region or AMR genes between *Salmonella* I 4,[5],12:i:- and publicly available *Salmonella* I 4,[5],12:i:- genomes isolated from swine and/or humans. As mentioned, *Salmonella* I 4,[5],12:i:- genomes were selected based on the Hierarchical Clustering of Core-genome (HierCC) MLST on Enterobase.

Salmonella I 4,[5],12:i:- (n = 701) were predominantly isolated from humans (67%); however, 33% were isolated from swine. Furthermore, *Salmonella* I 4,[5],12:i:- were primarily isolated in North America, specifically the United States (n = 635); whereas only 48 were isolated from European countries including Denmark (n = 5), Ireland (n = 5), Italy (n = 1), and the United Kingdom (n = 37). Three *Salmonella* I 4,[5],12:i:- sequence types (STs) were identified: 34, 2379, and 2956. The majority (85%) of the *Salmonella* I 4,[5],12:i:- genomes were ST34 (n = 578), followed by 2379 (n = 97), and 2956 (n = 8). Approximately 97% of *Salmonella* I 4,[5],12:i:- showed presence of the *iroB* gene and deletion of the *fljB,* and *fljA* genes which is similar to the *Salmonella* I 4,[5],12:i:- European clone. Only 12 genomes (~2%) showed the complete deletion of *iroB, fljB,* and *fljA* genes resembling the *Salmonella* I 4,[5],12:i:- Spanish clone (Table 2-

1). The deletion profile was not identified for ten of the genomes. Interestingly, those with the Spanish clone deletion were ST34, which is a MLST more frequently associated with the European clone deletion. The genomes with the Spanish clone deletion also exhibited a European clone resistance genotype harboring the resistance genes *bla*$_{\text{TEM-1}}$*, strA-strB, sul2,* and *tet*(B) which encodes resistance to ASSuT. Two of the twelve Spanish clone *Salmonella* I 4,[5],12:i:- were isolated from humans in Europe (Ireland and the United Kingdom), while the rest were isolated from human and swine in the United States. There were ten ST34 *Salmonella* I 4,[5],12:i:- genomes that were pan-susceptible similarly to the *Salmonella* I 4,[5],12:i:- United States clone and displayed the European clone deletion. Additionally, ~86% of the *Salmonella* I 4,[5],12:i:- genomes were MDR (n = 589). Most harbored genes known to provide resistance to ASSuT (n = 563) and showed similarity to the European clone resistance genotype (n = 554).

There were a total of eight ST2956 *Salmonella* I 4,[5],12:i:- that were classified as HC5_6 using HierCC MLST on Enterobase, along with 42 *Salmonella* I 4,[5],12:i:- isolates from our study, indicating a close relationship with only 5 cgMLST allelic differences. All ST2956 *Salmonella* I 4,[5],12:i:- were isolated from the United States and displayed the European deletion profile and resistance genotype (Figure 4-54). Three ST2956 *Salmonella* I 4,[5],12:i:- were isolated from swine while the other five were isolated from humans. One of the human ST2956 *Salmonella* I 4,[5],12:i:- strains additionally harbored the following genes: *aac*(3)-II, *aac*(6')-IIc, *aadA1, bla*$_{\text{SHV-12}}$,

catA1,ereA, sul1, and *sul2.* There was one ST34 *Salmonella* I 4,[5],12:i:- isolated from swine that was equally related to the ST2956 *Salmonella* I 4,[5],12:i:- strains. The eight ST2956 *Salmonella* I 4,[5],12:i:- and one ST34 *Salmonella* I 4,[5],12:i:- also shared a more recent common ancestor to six human and three swine ST34 *Salmonella* I 4,[5],12:i:- strains all of which harbored the *tet*(B) gene; two strains were non-MDR.

Within ST34 *Salmonella* I 4,[5],12:i:- strains, ST2379 emerges and forms its own clade suggesting it may have evolved from ST34 *Salmonella* I 4,[5],12:i:- (Figure 4-48). There are 97 ST2379 *Salmonella* I 4,[5],12:i:- strains which were also classified as HC5_6 along with 42 ST34 *Salmonella* I 4,[5],12:i:- isolates from our study and ST2956 *Salmonella* I 4,[5],12:i:- strains. All ST2379 *Salmonella* I 4,[5],12:i:- were shown to have the European deletion profile and have been isolated in the United States. Most ST2379 *Salmonella* I 4,[5],12:i:- were isolated from humans (n = 72) compared to swine (n = 26). The majority of ST2379 *Salmonella* I 4,[5],12:i:- also presented the European clone resistance genotype (n = 88). There were five ST2379*Salmonella* I 4,[5],12:i:- that further formed a clade and displayed a distinct MDR resistance pattern, with the resistance genes *aadA1, sul1,* and *tet*(B), with four also having the *aac*(3)-VIa gene. There were two human ST2379 *Salmonella* I 4,[5],12:i:- that were not multidrug-resistant with one being pan-susceptible and the other harboring only the *tet*(B) gene. Furthermore, ST2379 *Salmonella* I 4,[5],12:i:- strains shared a common ancestor with 12 ST34 *Salmonella* I 4,[5],12:i:- strains isolated from the United States that showed the European clone resistance

genotype. Of these 12 genomes, eleven displayed the European clone deletion profile while one showed the Spanish clone deletion profile. There were seven closely related ST34 *Salmonella* I 4,[5],12:i:- (two human and five swine isolates) that showed presence of multiple resistance genes, including *aac*(3)-II, *aac*(6')-Ib, *aac*(6')-IIc, *aadA1, aadA2, aph*-(3')-Ia, *aph*-(3')-Ib, $bla_{\text{SHV-12}}$, *catA1, dfrA19, ereA, mcr*-9*, qnrB*2, *sul1,* and *tet*(D).

In Figure 4-48, a subtree of all *Salmonella* I 4,[5],12:i:- genomes are shown with nodes labeled according to sequence type. The sequence type of *Salmonella* I 4,[5],12:i:- had a major influence on the genetic relatedness of genomes. Although the majority were ST34, sequence types ST2379 (Figure 4-54) and ST2956 (Figure 4-55) each clustered together and formed individual clades with other closely related ST34 genomes. Moreover, the location where the *Salmonella* I 4,[5],12:i:- were isolated also tended to form one monophyletic group such as most European (Denmark, Ireland, United Kingdom) *Salmonella* I 4,[5],12:i:- clustered together. On the other hand, source type (human or swine) did not have a large impact on genetic relatedness of *Salmonella* I 4,[5],12:i:- genomes.

Of the 46 *Salmonella* I 4,[5],12:i:- in our study, 20 were found in subtree 1 (Figure 4-50). Nineteen were isolated in September and one was isolated in November. While most of the *Salmonella* I 4,[5],12:i:- in subtree 1were genomes from our study, there were four other closely related *Salmonella* I 4,[5],12:i:- that were isolated from humans in the United States. The four isolates all showed the ASSuT genotype (MDR) commonly

associated with the European clone. Additionally, these isolates showed the European clone deletion profile. None harbored the *aadA* gene as the other isolates in subtree 1 and only one additionally harbored the *qnrB*19 gene.

In Figure 4-51 (subtree 2), one *Salmonella* I 4,[5],12:i:- from our study that was isolated in January clustered with 28 publicly available *Salmonella* I 4,[5],12:i:- genomes. All of the *Salmonella* I 4,[5],12:i:- displayed the European clone deletion profile. The one isolate from our study shared a recent common ancestor with two *Salmonella* I 4,[5],12:i:-, one isolated from swine and another isolated from a human in the United States. Only the swine *Salmonella* I 4,[5],12:i:- genome displayed the European clone resistance type (ASSuT), while the human *Salmonella* I 4,[5],12:i:- genome only showed presence of the *tet*(B) resistance gene. Furthermore, two additional *Salmonella* I 4,[5],12:i:- were found to be equally related to the three isolates mentioned before (the one from our study, the swine and human genome). These isolates were isolated from swine in the United States and exhibited the European clone resistance genotype with one having the additional resistance gene *aph*(3')-Ia. The remaining *Salmonella* I 4,[5],12:i:- in the cluster include closely related isolates from humans (n = 14) and swine (n = 9). There were seven *Salmonella* I 4,[5],12:i:- that did not present the ASSuT genotype; however, three closely related isolates forming a monophyletic group were still MDR harboring $bla_{\text{TEM-1}}$, *strA-B, sul2,* and *aph*(3')-Ia. While the majority were isolated in the United States, there were four

human *Salmonella* I 4,[5],12:i:- that were isolated in Europe from the United Kingdom, which also presented the European clone resistance genotype.

In subtree 3 (Figure 4-52), 11 *Salmonella* I 4,[5],12:i:- from our study clustered with 15 other *Salmonella* I 4,[5],12:i:- genomes. The 11 *Salmonella* I 4,[5],12:i:- from our study were isolated during the months of March, May, and November. One *Salmonella* I 4,[5],12:i:- from our study that was isolated in March formed a clade with two other *Salmonella* I 4,[5],12:i:-, one isolated from a human and one from swine in the United States. The two isolates were also MDR and presented the European clone resistance genotype. This clade was closely related to another human *Salmonella* I 4,[5],12:i:- isolated in the United States which showed the European clone resistance genotype and had the *qnrB*19 gene. There were three *Salmonella* I 4,[5],12:i:- isolates in our study from November that harbored additional resistance genes such as *aph*-(3')-Ia, $bla_{\text{SHV-12}}$, *dfrA19, mcr*-9*, qnrB*2, *sul1,* and *tet*(D). Interestingly, these MDR *Salmonella* I 4,[5],12:i:- formed a clade with one *Salmonella* I 4,[5],12:i:- isolated from swine in the United States which harbored identical resistance genes. This one *Salmonella* I 4,[5],12:i:- genome additionally, harbored the *aac*(3)-II, *aac*(6')-IIc, *aadA2*, *ereA*, and *floR* resistance genes. The *Salmonella* I 4,[5],12:i:- in this clade are related to one human *Salmonella* I 4,[5],12:i:- isolate from the United States showing the European clone resistance genotype and three *Salmonella* I 4,[5],12:i:- isolates from our study (two isolated in May and one in March). Furthermore, three *Salmonella* I 4,[5],12:i:- isolates in our study isolated in March

were closely related to numerous *Salmonella* I 4,[5],12:i:- isolated from swine in the United States including one harboring the European clone resistance genotype and several additional resistance genes (*aac*(3)-II, *aac*(3)-IIc, *aadA1, aadA2, aph*-(3')-Ia, *catA2*, *dfrA19, ereA, mcr*-9*, qnrB*2, and *sul1*). Lastly, one *Salmonella* I 4,[5],12:i:- from our study (isolated in May) was closely related to five swine and one human *Salmonella* I 4,[5],12:i:- from the United States. Four of the five swine *Salmonella* I 4,[5],12:i:- harbored multiple resistance genes including *aac*(3)-II, *aac*(3)-IId, *aac*(3)-VIa, *aac*(6')-Ib, *aac*(6')-IIc, *aadA1, aadA2, aph*-(3')-Ia, *dfrA14, dfrA15, dfrA19, ereA, mcr*-9*, qnrB*19, *qnrB*77, and *sul1.* Three *Salmonella* I 4,[5],12:i:- that harbored *dfrA14* and *qnrB19* were further closely related to each other.

In Figure 4-53, subtree 4 shows 14 *Salmonella* I 4,[5],12:i:- genomes from our study and several closely related and publicly available *Salmonella* I 4,[5],12:i:- genomes isolated from the United States. This subtree consists primarily of swine *Salmonella* I 4,[5],12:i:- strains but includes both non-MDR and MDR genotypes. Most isolates in this clade, with the exception of two, harbored the *tet*(B) resistance gene. Thirteen *Salmonella* I 4,[5],12:i:- harboring *qnrB*19 and isolated in November from our study further formed a clade and shared a common ancestor with a *Salmonella* I 4,[5],12:i:- strain isolated from a human. This human *Salmonella* I 4,[5],12:i:- strain presented the European deletion profile and resistance genotype and harbored the *qnrB*19 gene. This clade was also closely related to other swine and human strains including the clade of 14 *Salmonella* I 4,[5],12:i:-

from our study. Additionally, non-MDR *Salmonella* I 4,[5],12:i:- strains isolated from swine further formed a clade with three MDR *Salmonella* I 4,[5],12:i:- strains showing the European deletion profile and resistance genotype. Interestingly, in a separate clade, there was one MDR human *Salmonella* I 4,[5],12:i:- isolate with the United States deletion profile. This isolate did not display a ASSuT genotype as it lacked the *tet*(B) gene; however, it harbored the *qnrB*19 gene.

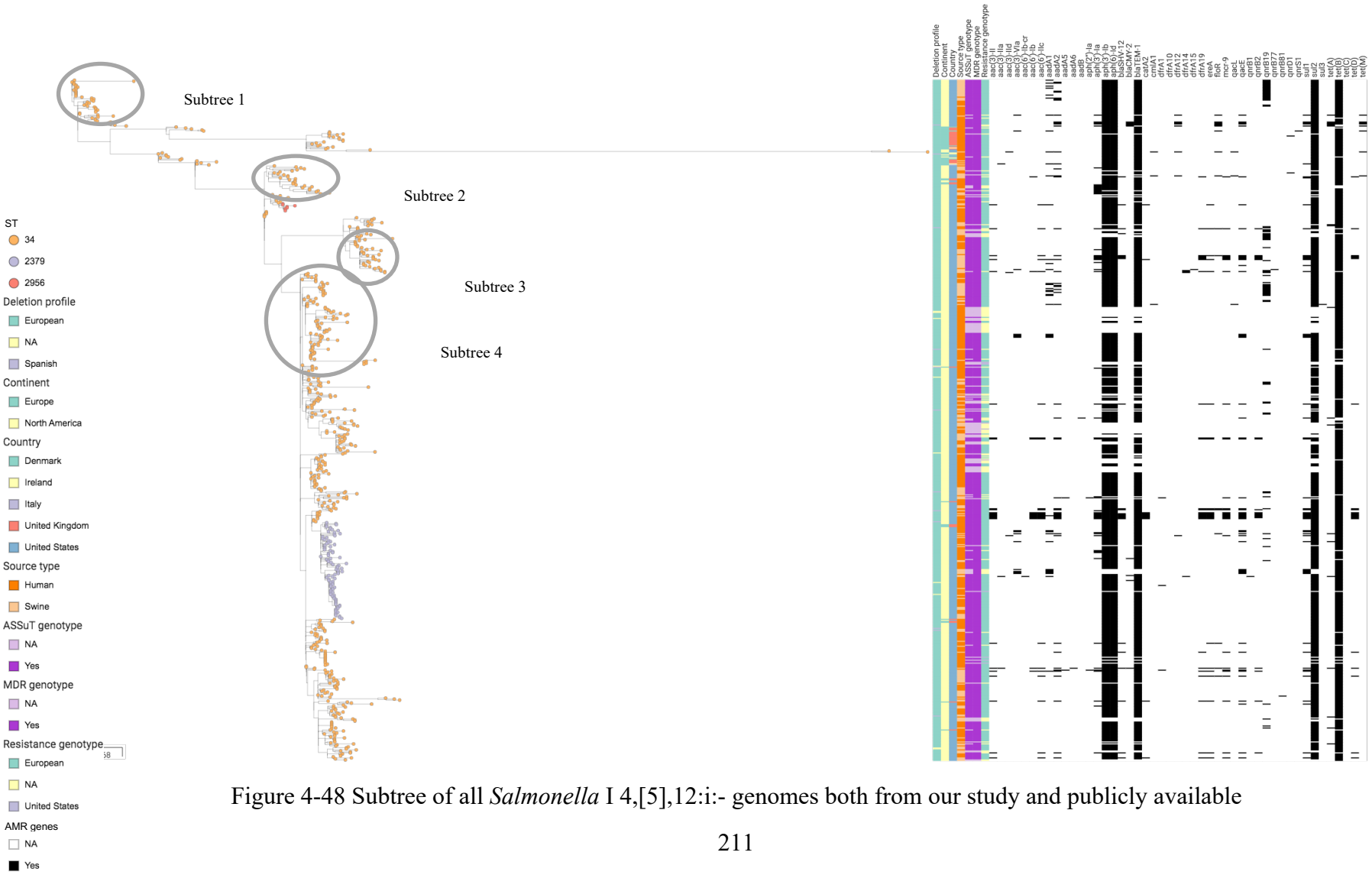


Figure 4-48 Subtree of all *Salmonella* I 4,[5],12:i:- genomes both from our study and publicly available

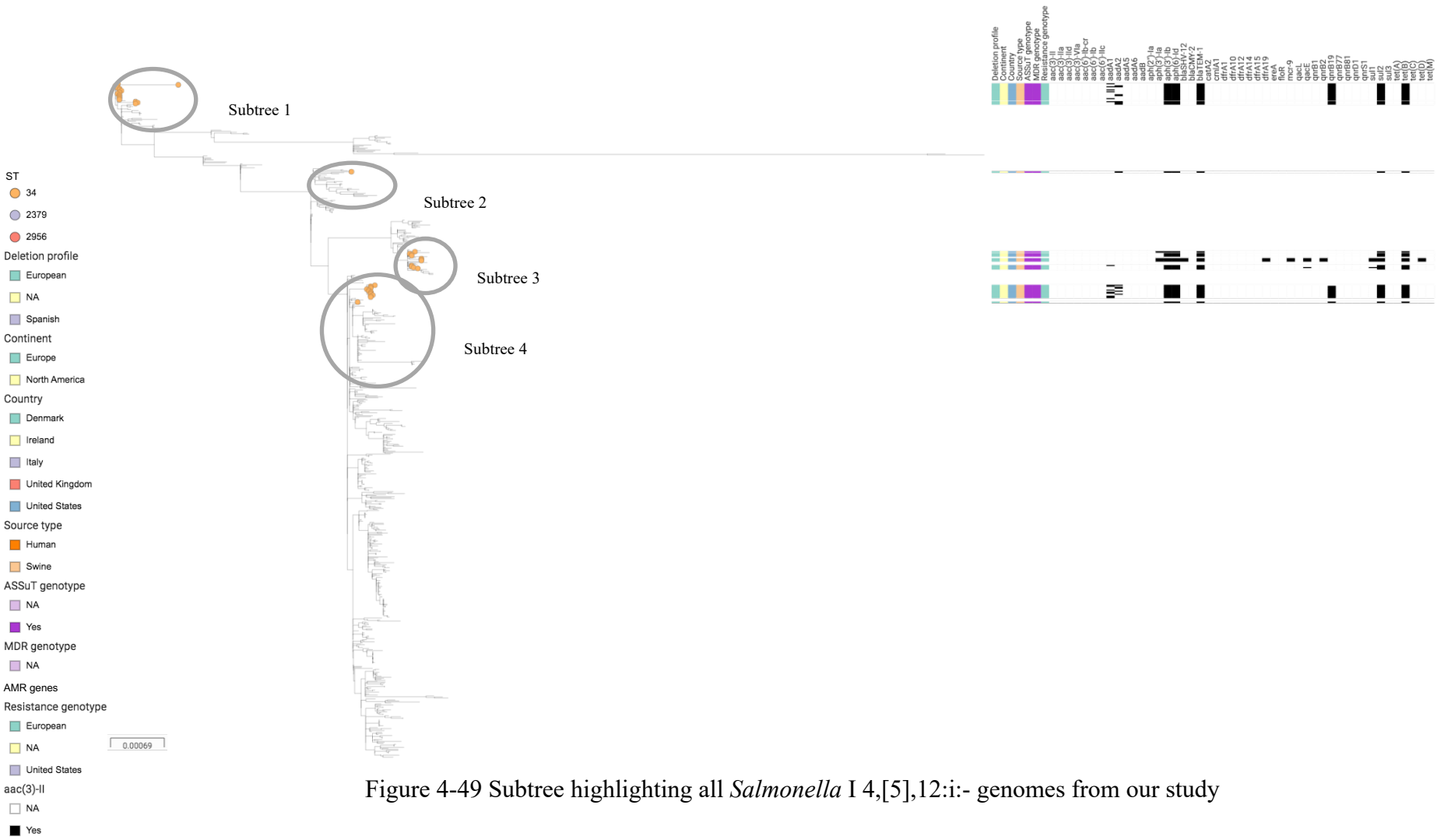
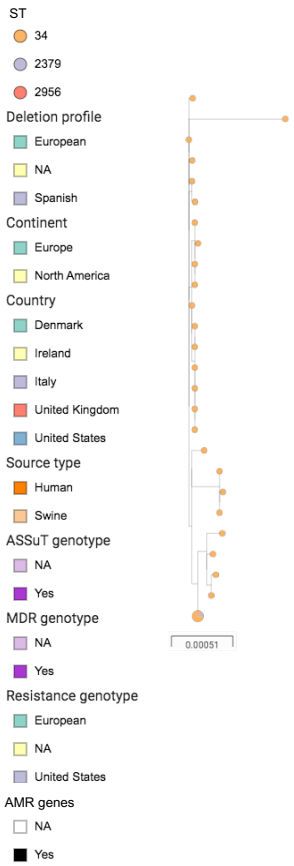


Figure 4-49 Subtree highlighting all *Salmonella* I 4,[5],12:i:- genomes from our study



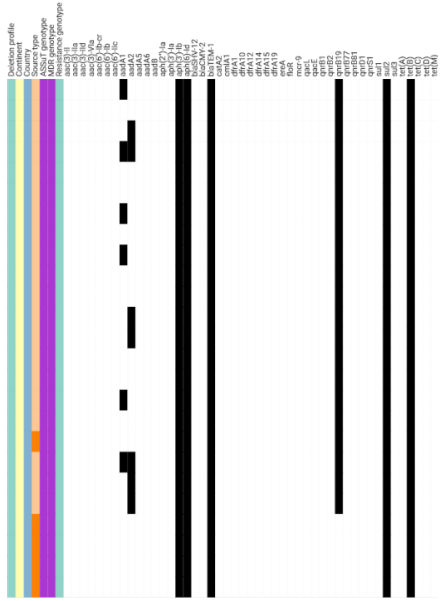


Figure 4-50 Subtree 1 of ST34 *Salmonella* I 4,[5],12:i:- genomes

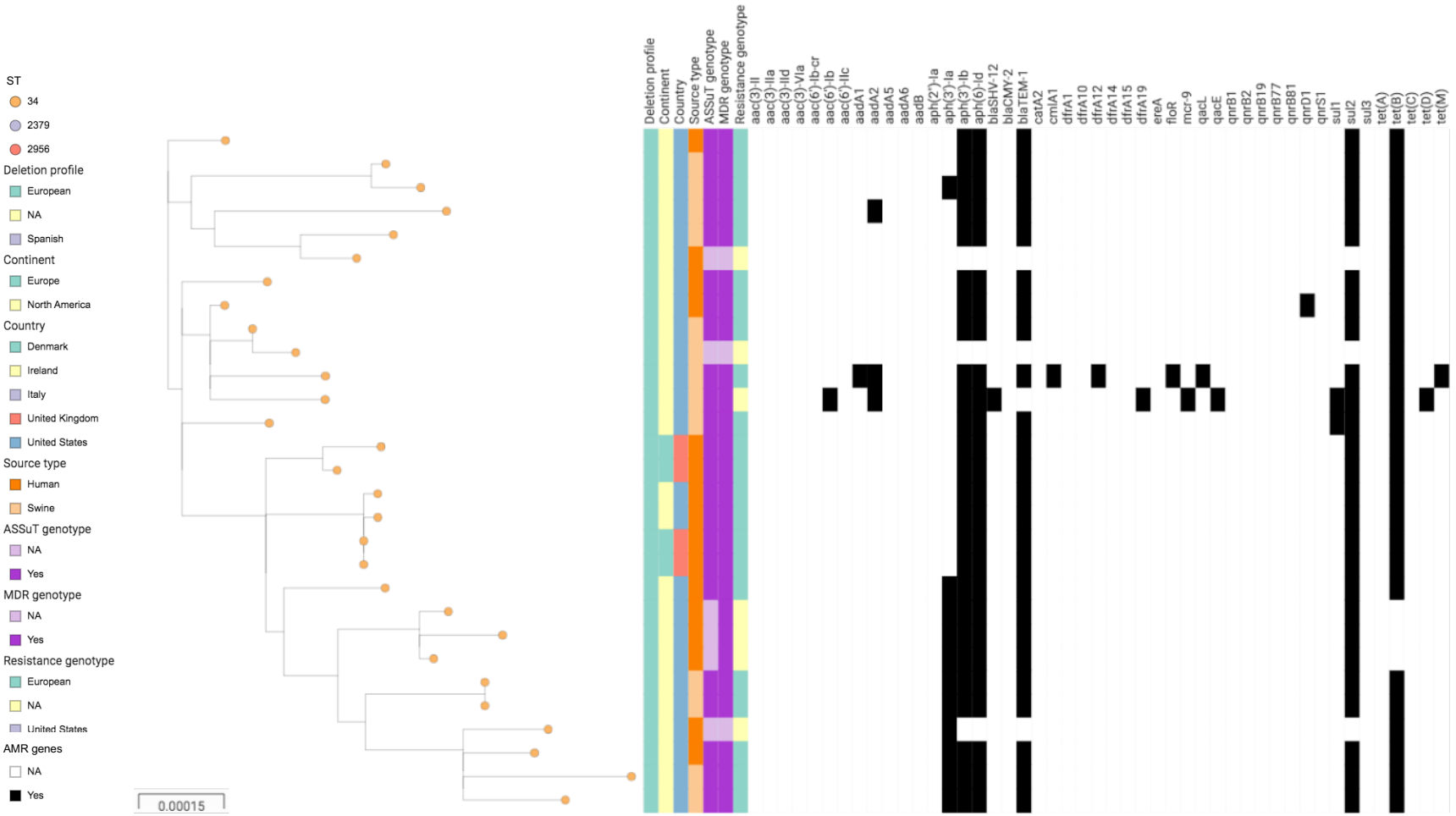


Figure 4-51 Subtree 2 of ST34 *Salmonella* I 4,[5],12:i:- genomes

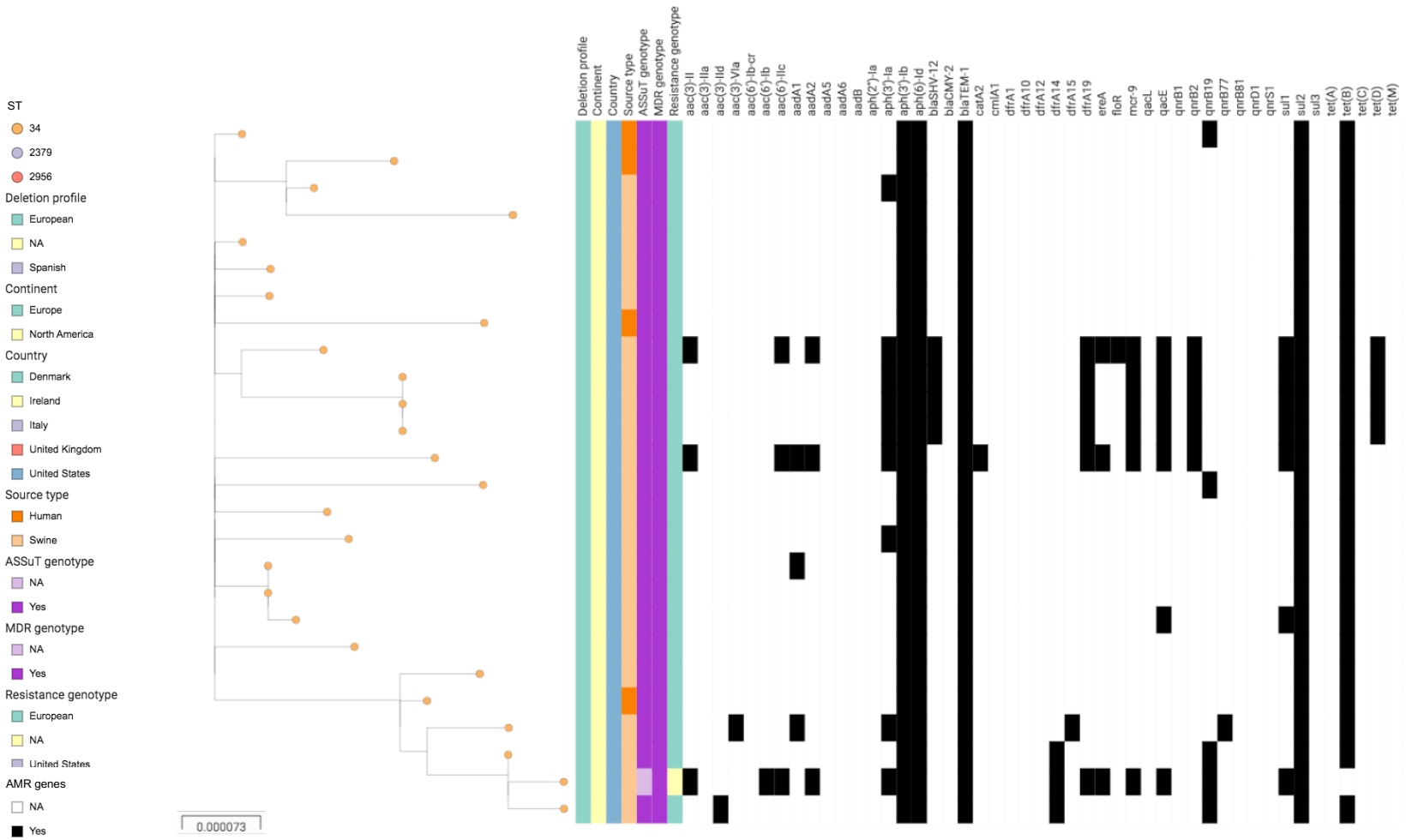


Figure 4-52 Subtree 3 of ST34 *Salmonella* I 4,[5],12:i:- genomes

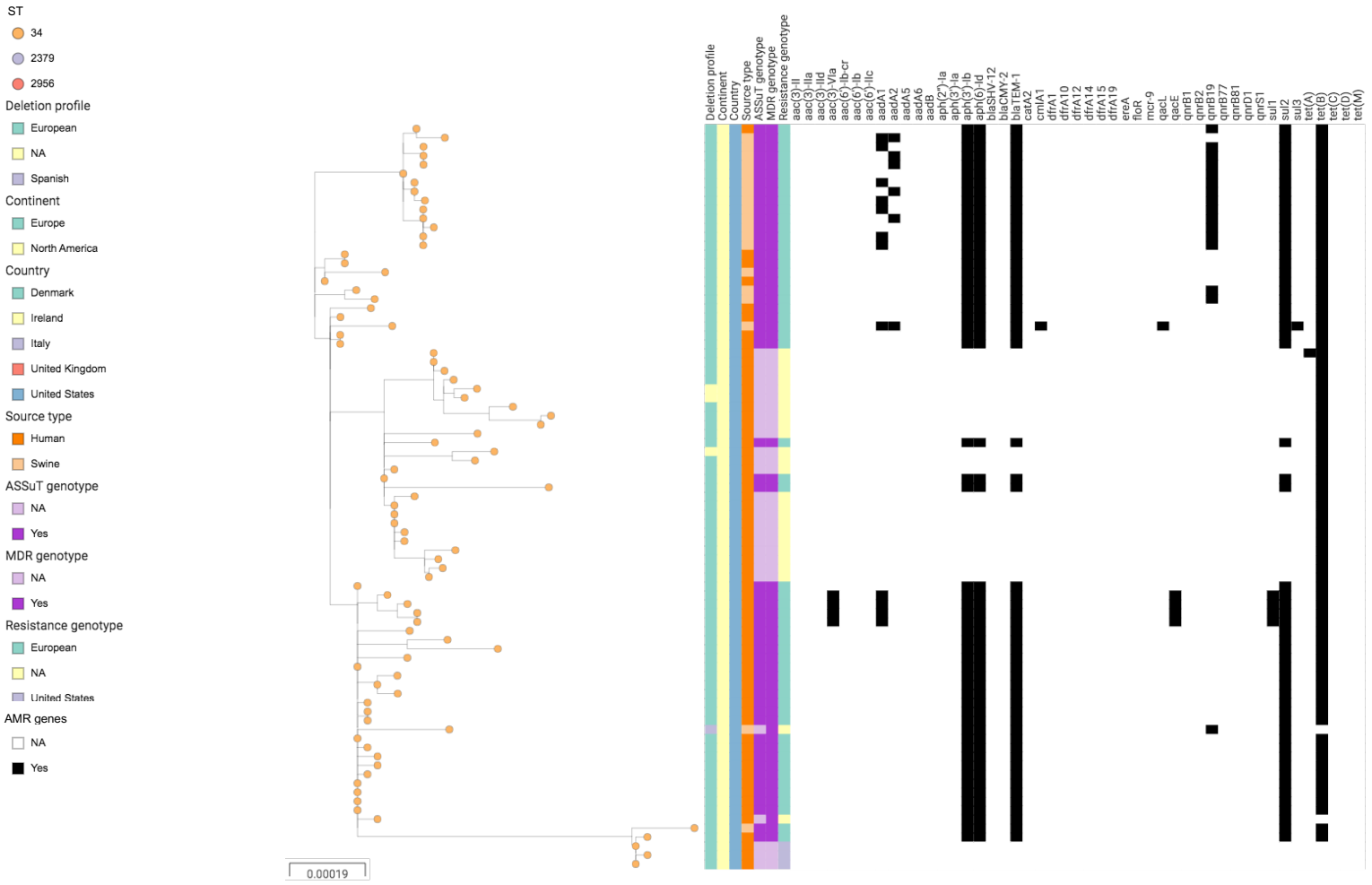


Figure 4-53 Subtree 4 of ST34 *Salmonella* I 4,[5],12:i:- genomes

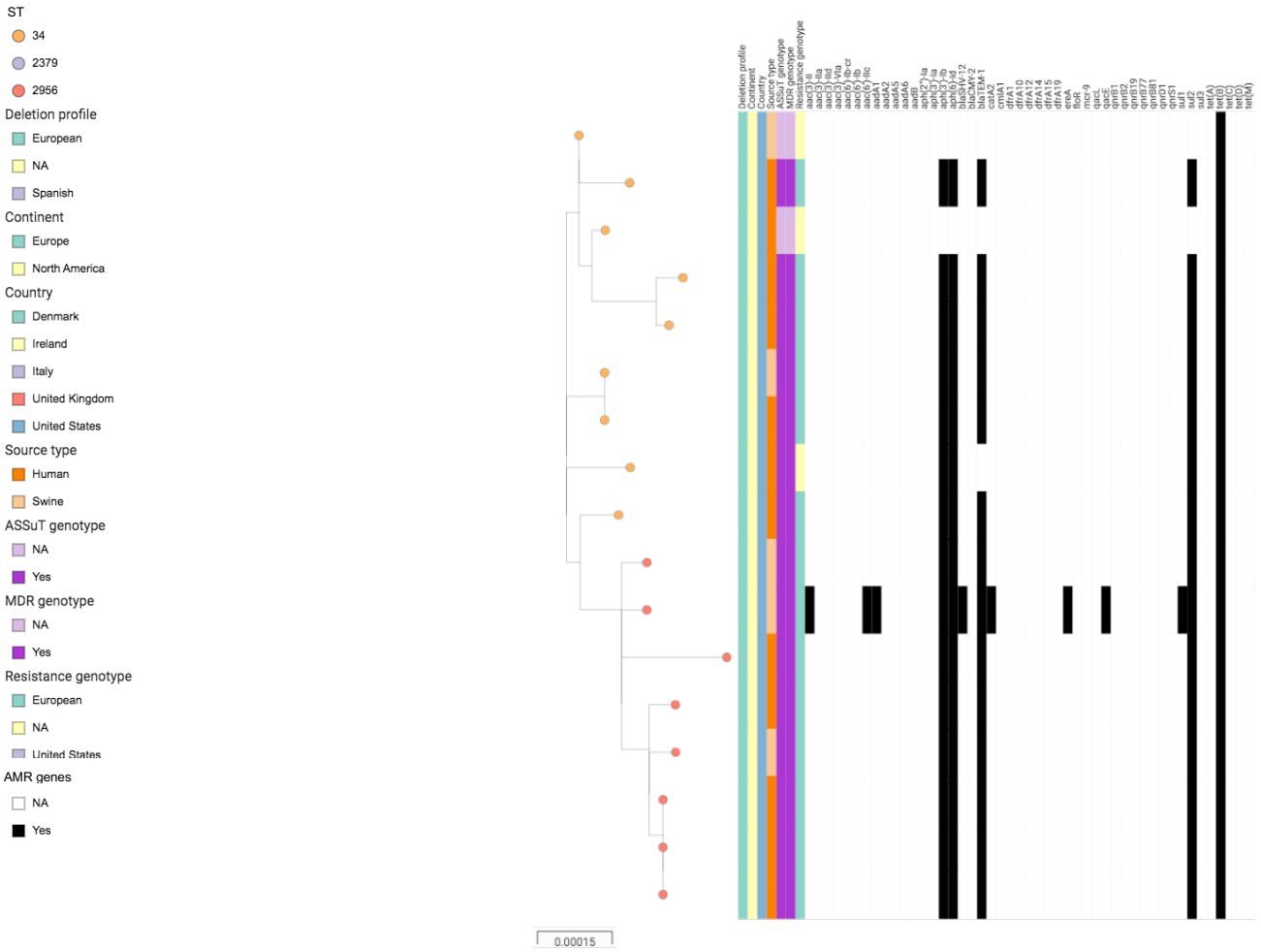


Figure 4-54 Subtree of ST2956 *Salmonella* I 4,[5],12:i:- genomes

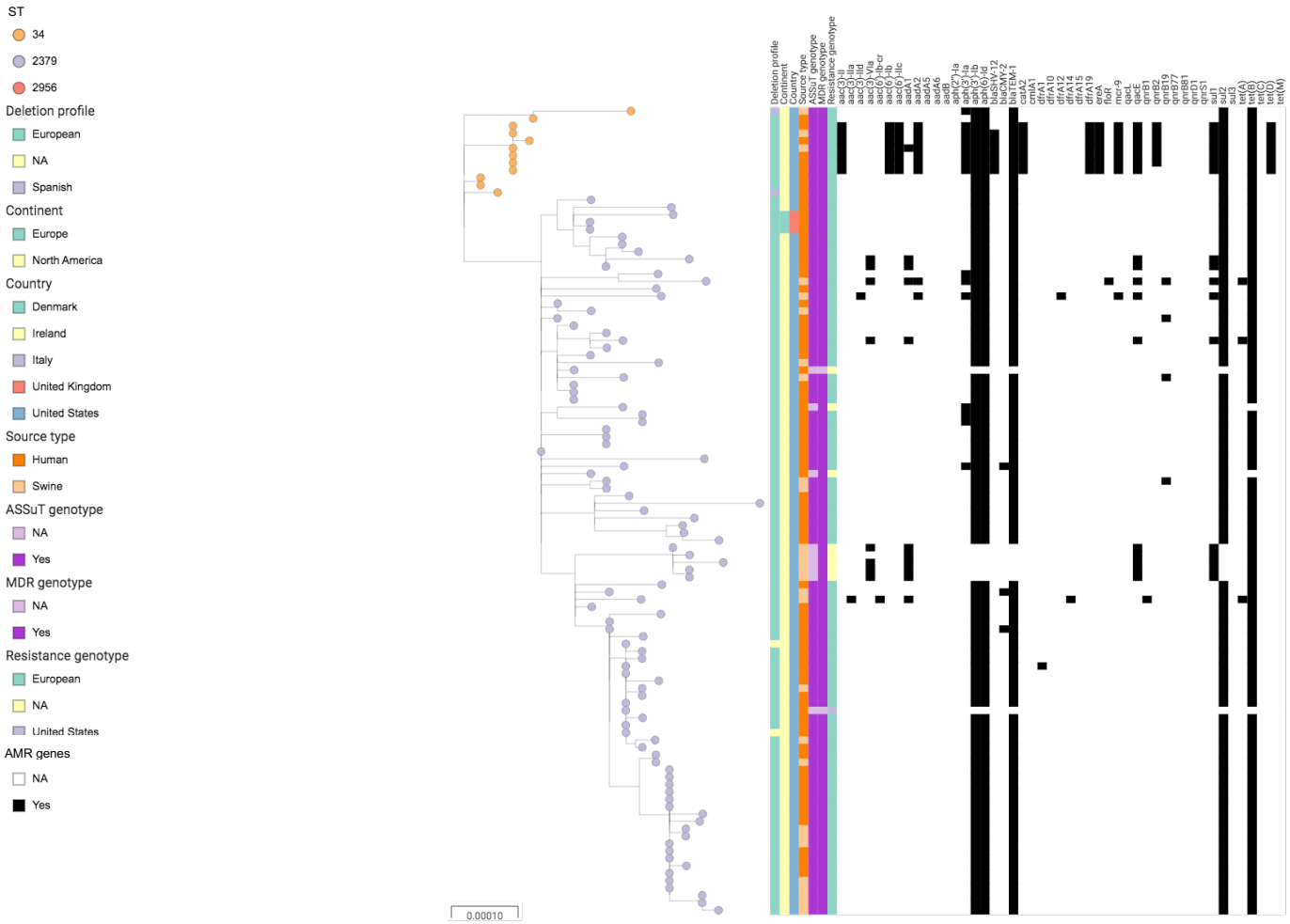


Figure 4-55 Subtree of ST2379 *Salmonella* I 4,[5],12:i:- genomes

4.8.3. All Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

In addition to the 701 *Salmonella* I 4,[5],12:i:- genomes (publicly available and from our study) and 38 *Salmonella* Typhimurium from our study, 208 publicly available *Salmonella* Typhimurium genomes were selected based on the least allelic differences (HeirCC) to the *Salmonella* I 4,[5],12:i:- isolates from our study. A third phylogenetic analysis was conducted with the 701 *Salmonella* I 4,[5],12:i:- genomes and 246 *Salmonella* Typhimurium genomes to determine evolutionary relationships between the two serovars.

Three sequence types were identified among the *Salmonella* Typhimurium genomes: ST19, ST213, and ST34. The majority of the *Salmonella* Typhimurium genomes were ST19 (n = 224), followed by ST213 (n = 13) and ST34 (n = 3). There was one *Salmonella* Typhimurium that was a novel ST. All *Salmonella* Typhimurium were from the United States and isolated from swine. Approximately 86% of the *Salmonella* Typhimurium were MDR (n = 211) with most displaying the ASSuT genotype (n = 185; ~88%). Seventeen *Salmonella* Typhimurium had one or two resistance genes present such as *aadA2, bla*TEM-1, *sul1*, and *tet*(B) while 13 had no resistance genes.

Figure 4-56 shows a phylogenetic tree of *Salmonella* I 4,[5],12:i:- (n = 701) and *Salmonella* Typhimurium (n = 246) genomes with the reference genome *Salmonella* Enteritidis str. 18569. To take a closer look into the clades, a subtree was created without the reference genome (Figure 4-57). In Figure 4-57, the *Salmonella* strains are clearly

grouped by their sequence type as well as their respective serovar. The majority of ST19 *Salmonella* Typhimurium formed an individual clade and a few smaller monophyletic groups; a second clade was formed from the same node with all the ST213 *Salmonella* Typhimurium, three ST19 *Salmonella* Typhimurium, and all the *Salmonella* I 4,[5],12:i:- genomes. Interestingly, ST213 *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- shared a more recent common ancestor than ST19 *Salmonella* Typhimurium. As before, *Salmonella* I 4,[5],12:i:- sequence types formed individual clades.

When broadly looking at the subtree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes, major differences are observed in the resistance patterns of each serovar. While *Salmonella* I 4,[5],12:i:- commonly displayed the European clone resistance profile, *Salmonella* Typhimurium primarily showed presence of *aadA1, aadA2,* $bla_{\text{CARB-2}}$, *floR, sul1, tet*(A), and *tet*(G). Additional resistance genes were also present in the *Salmonella* Typhimurium isolates: *aph*(3')-Ia, *strA-B*, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, *cmlA1, dfrA12, sul2, sul3,* and *tet*(M). A few *Salmonella* Typhimurium isolates harbored the *qnr*B2 gene (n = 4) and *qnr*B19 (n = 8) genes. Figure 4-58 highlights all *Salmonella* Typhimurium genomes in the tree in comparison to the *Salmonella* I 4,[5],12:i:- and Figure 4-59 is a subtree of ST19 and ST213 *Salmonella* Typhimurium. There were distinctions in AMR genes between two ST19 *Salmonella* Typhimurium clades (Figure 4-59). In the first clade, most ST19 *Salmonella* Typhimurium did not exhibit a ASSuT genotype and were non-MDR strains. In contrast, the second ST19 *Salmonella*

Typhimurium clade largely consisted of MDR strains and displayed a ASSuT genotype. Although the *aph*(3')-la, *bla*$_{\text{TEM-1}}$, *strA-B*, *sul2*, and *tet*(B) resistance genes were found throughout ST19 *Salmonella* Typhimurium strains, they were predominantly present in the first ST19 clade. Similarly, *aadA2, bla*$_{\text{CARB-2}}$, *dfrA12, floR, sul1,* and *sul3* were predominantly found in the second ST19 clade. ST19 *Salmonella* Typhimurium in the second clade were the only strains to harbor the *tet*(G) and *tet*(M) resistance genes. The *aadA1* and *tet*(A) resistance genes were frequently found throughout both ST19 *Salmonella* Typhimurium clades. *Salmonella* Typhimurium from our study were mainly in the first clade (n = 26) compared to the second clade (n = 11) (Figure 4-60).

ST213 *Salmonella* Typhimurium were found to form a clade with three ST19 *Salmonella* Typhimurium and with all *Salmonella* I 4,[5],12:i:-. In this clade, *Salmonella* Typhimurium were all MDR and showed a ASSuT genotype and harbored the *tet*(A) resistance gene. The three ST19 *Salmonella* Typhimurium showed a slightly different resistance pattern than the other two ST19 as they harbored *aadA7, strA-B*, *bla*$_{\text{CMY-2}}$, *floR, sul1, sul2,* and *tet*(B). Twelve of ST213 *Salmonella* Typhimurium additionally harbored the *aph*(3')-Ia and *bla*$_{\text{TEM-1}}$ genes, while 11 closely related ST213 *Salmonella* Typhimurium and harbored the *aadA12* and *sul1* genes. Furthermore, there were two related ST213 *Salmonella* Typhimurium that harbored the *bla*$_{\text{CMY-2}}$, *strA-B, floR,* and *sul2 genes* and one showed the presence of the *qnrB*19 gene while the other the *tet*(B) gene. Extending from the same node as the ST213 *Salmonella* Typhimurium, are *Salmonella* I

4,[5],12:- isolates. This suggests ST213 *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:- originate from the same ancestral lineage and have more recent common ancestors than ST19 *Salmonella* Typhimurium.

Lastly, there were three ST34 *Salmonella* Typhimurium strains isolated from swine in the United States which were closely related to human ST34 *Salmonella* I 4,[5],12:i:- from European countries (e.g. the United Kingdom and Ireland), exhibiting the European clone resistance genotype and are in the same clade as one isolate from Italy (Figure 4-62). In addition to genotypic serotyping, the first and second phase flagellar antigen genes (*fliC* and *fljB*) were present along with genes involved in flagellar phase variation (*iroB* and *fljA*). All three ST34 *Salmonella* Typhimurium harbored *strA-B*, and *sul2* genes while two also had the $bla_{\text{TEM-1}}$ and *tet*(B) gene. One ST34 *Salmonella* Typhimurium harbored several more genes including *aadA1,* $bla_{\text{CMY-2}}$, *cmlA*, *floR, sul1,* and *tet*(A). Unlike other ST19 and ST213 *Salmonella* Typhimurium, *tet*(G) and *tet*(M) resistance genes were not found.

4.8.3.1. Timeline of Salmonella *I 4,[5],12:i:- and* Salmonella *Typhimurium*

Figure 4-63 shows a timeline of isolation of the *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes included in the phylogenetic analyses between the year 2001 and 2021. The dots represent the genomes and are colored by sequence type. It can be observed that ST19 *Salmonella* Typhimurium and ST213 *Salmonella* Typhimurium were largely isolated before 2011. However, isolation of ST19 and ST213

continues every year with many being isolated in 2015. ST34 *Salmonella* Typhimurium was isolated in 2017 and 2019. The first appearance of ST34 *Salmonella* I 4,[5],12:i:- genomes included in this study was in 2007. ST34 *Salmonella* I 4,[5],12:i:- gradually started to increase with most isolated between 2015 and 2017. The first isolation of the selected ST2379 *Salmonella* I 4,[5],12:i:- was in 2013 and while ST2379 has been isolated the following years, the majority were isolated in 2015 and 2016. ST2956 *Salmonella* I 4,[5],12:i:- were evenly distributed between 2015 and 2018.

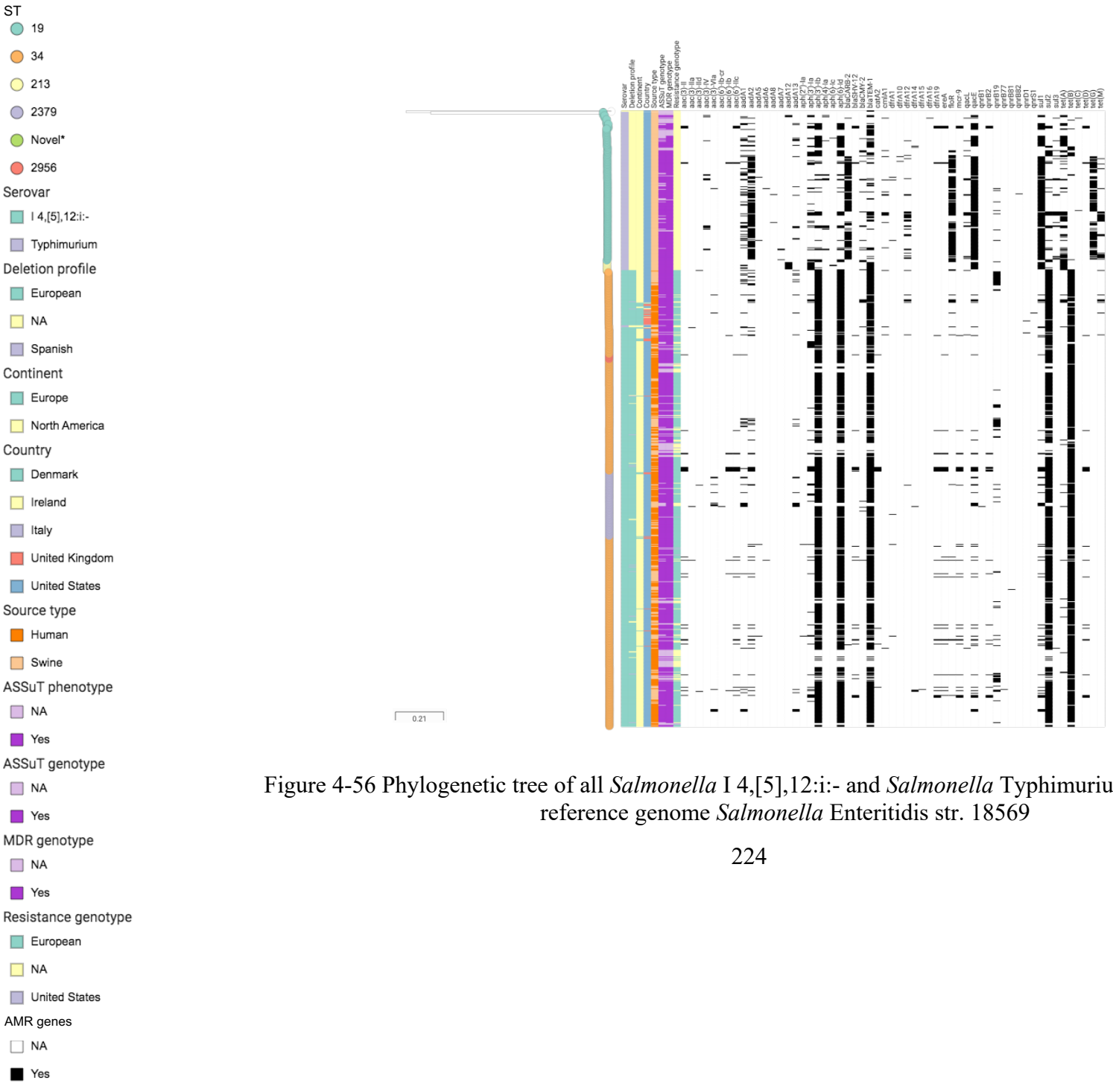


Figure 4-56 Phylogenetic tree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes with the reference genome *Salmonella* Enteritidis str. 18569

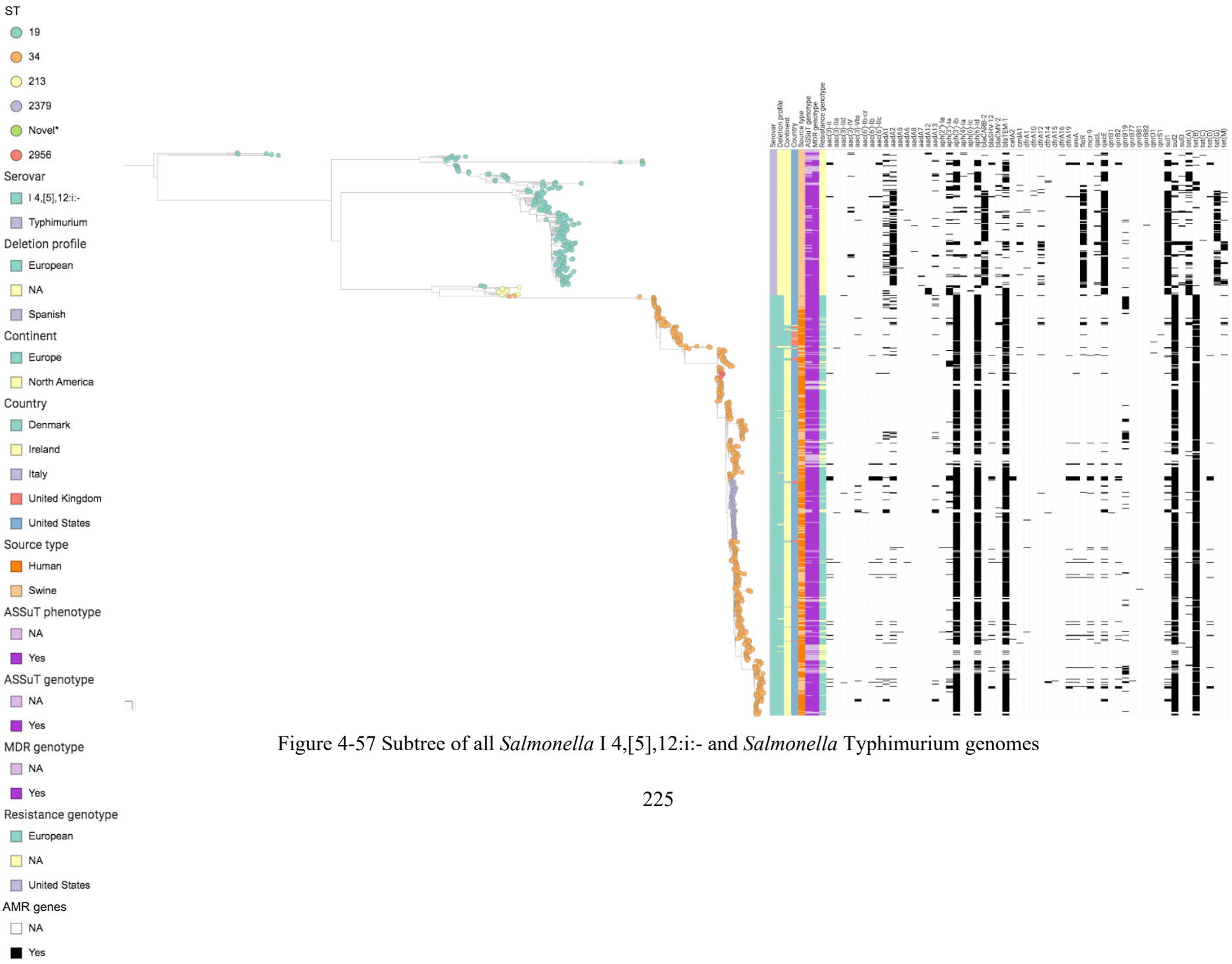


Figure 4-57 Subtree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes

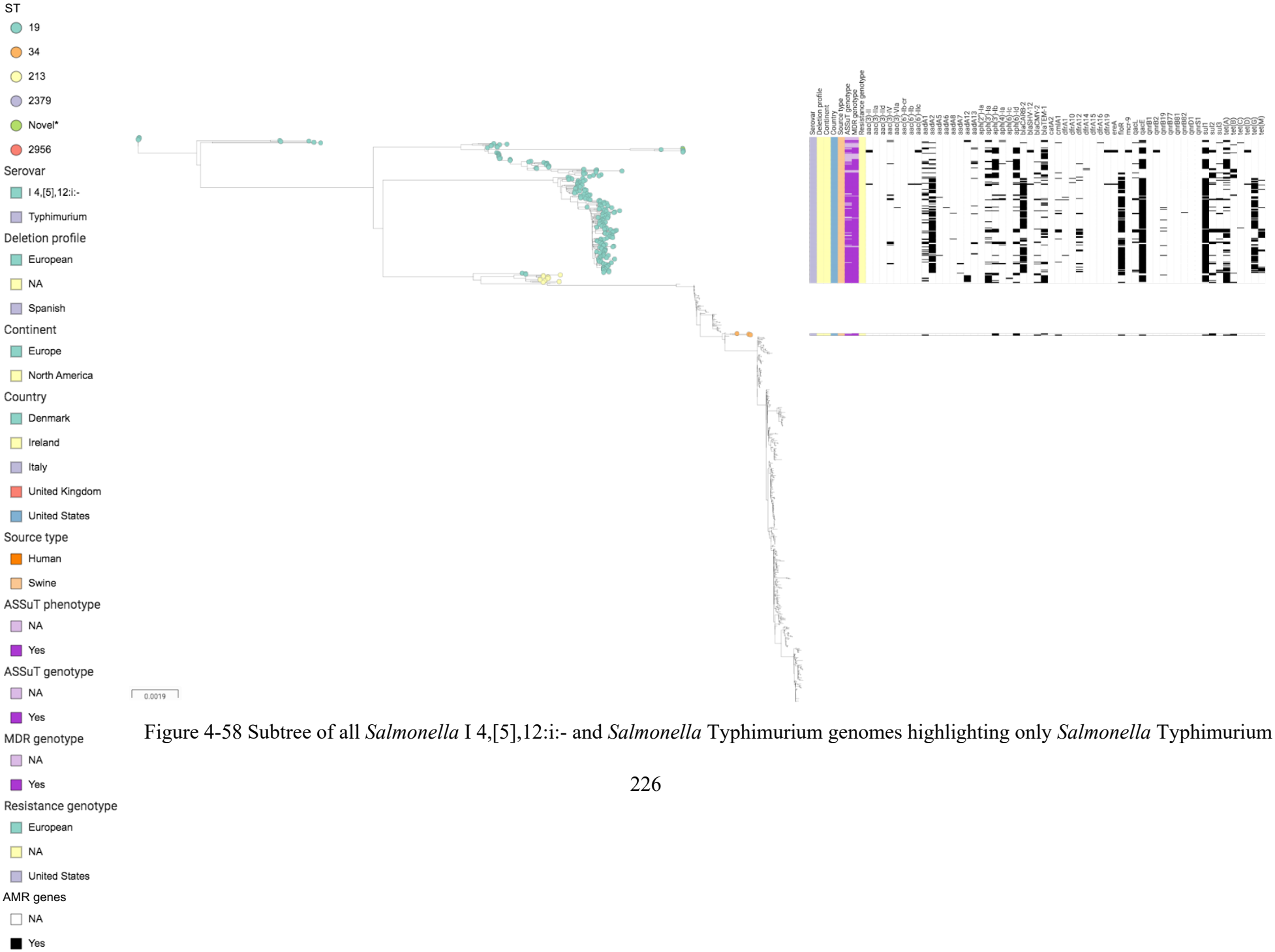


Figure 4-58 Subtree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes highlighting only *Salmonella* Typhimurium

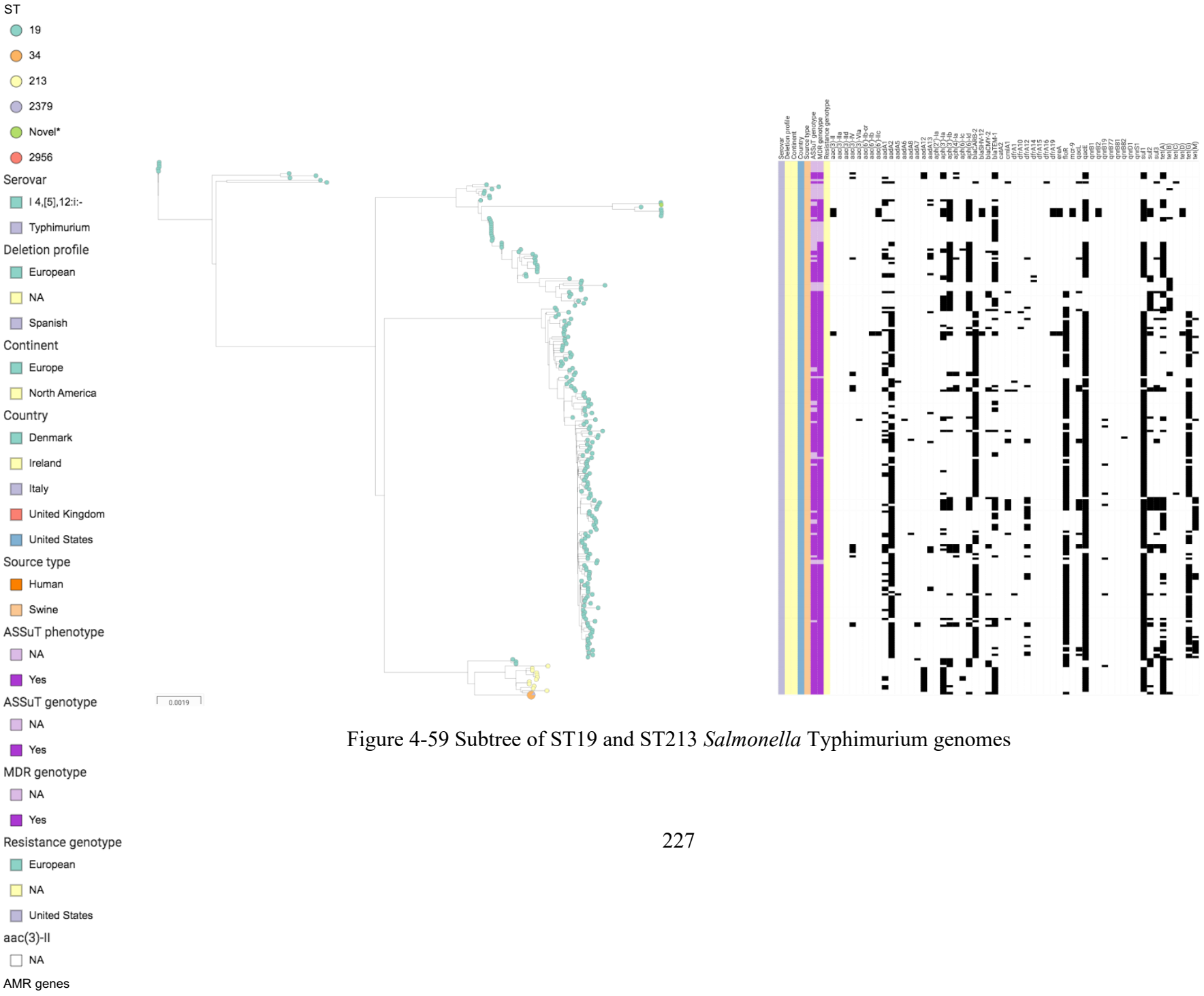


Figure 4-59 Subtree of ST19 and ST213 *Salmonella* Typhimurium genomes

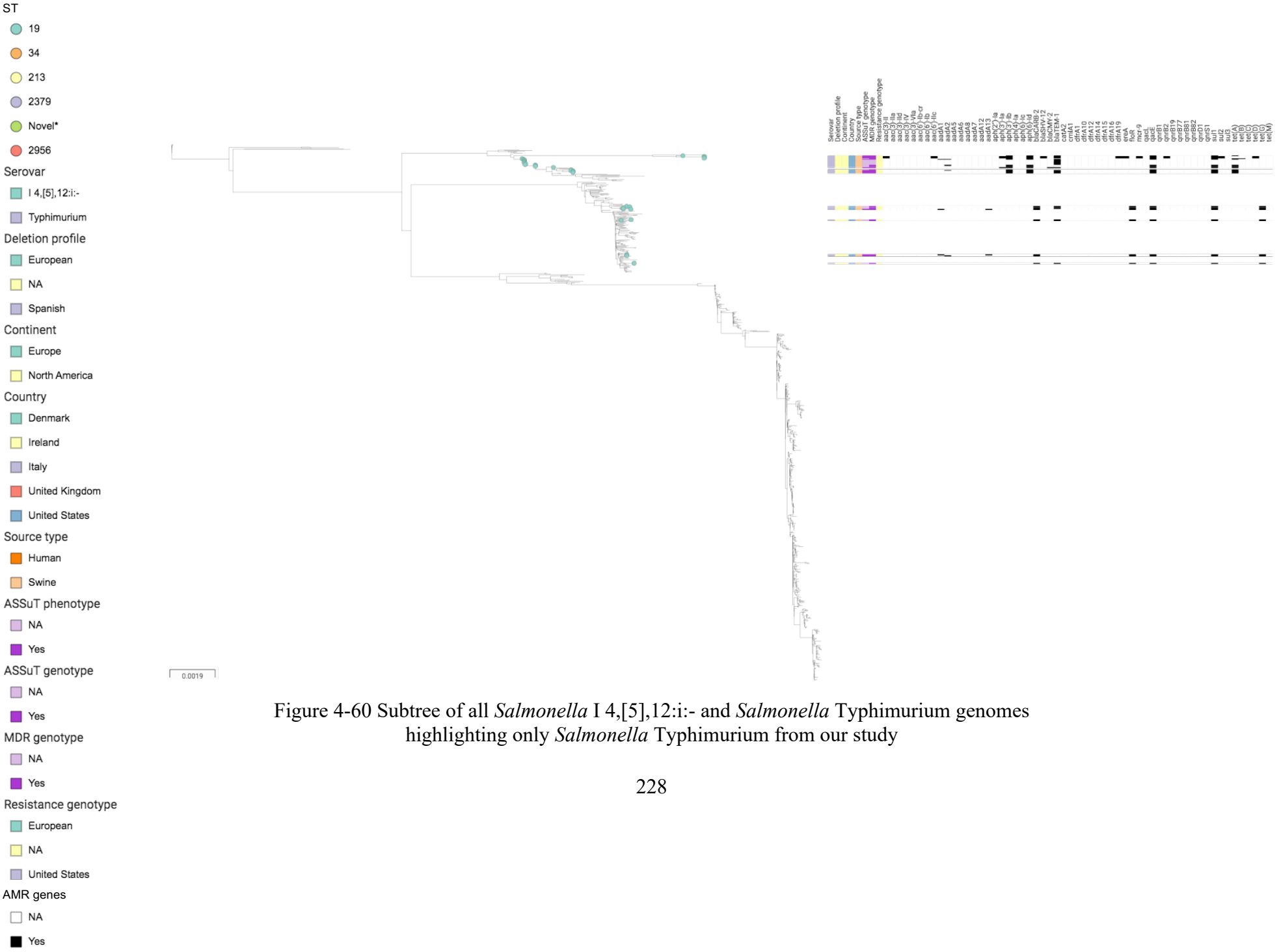


Figure 4-60 Subtree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes highlighting only *Salmonella* Typhimurium from our study

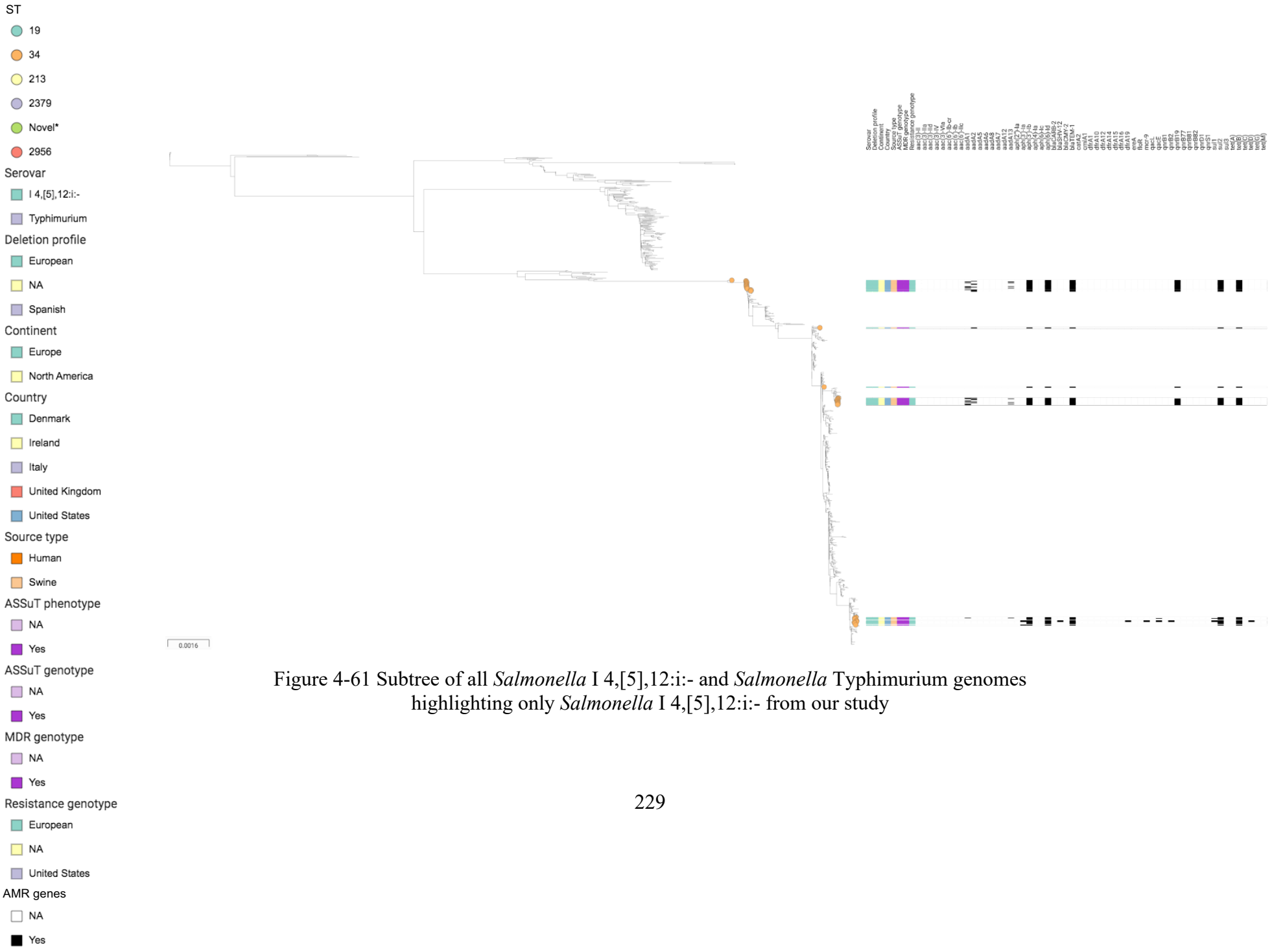


Figure 4-61 Subtree of all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes highlighting only *Salmonella* I 4,[5],12:i:- from our study

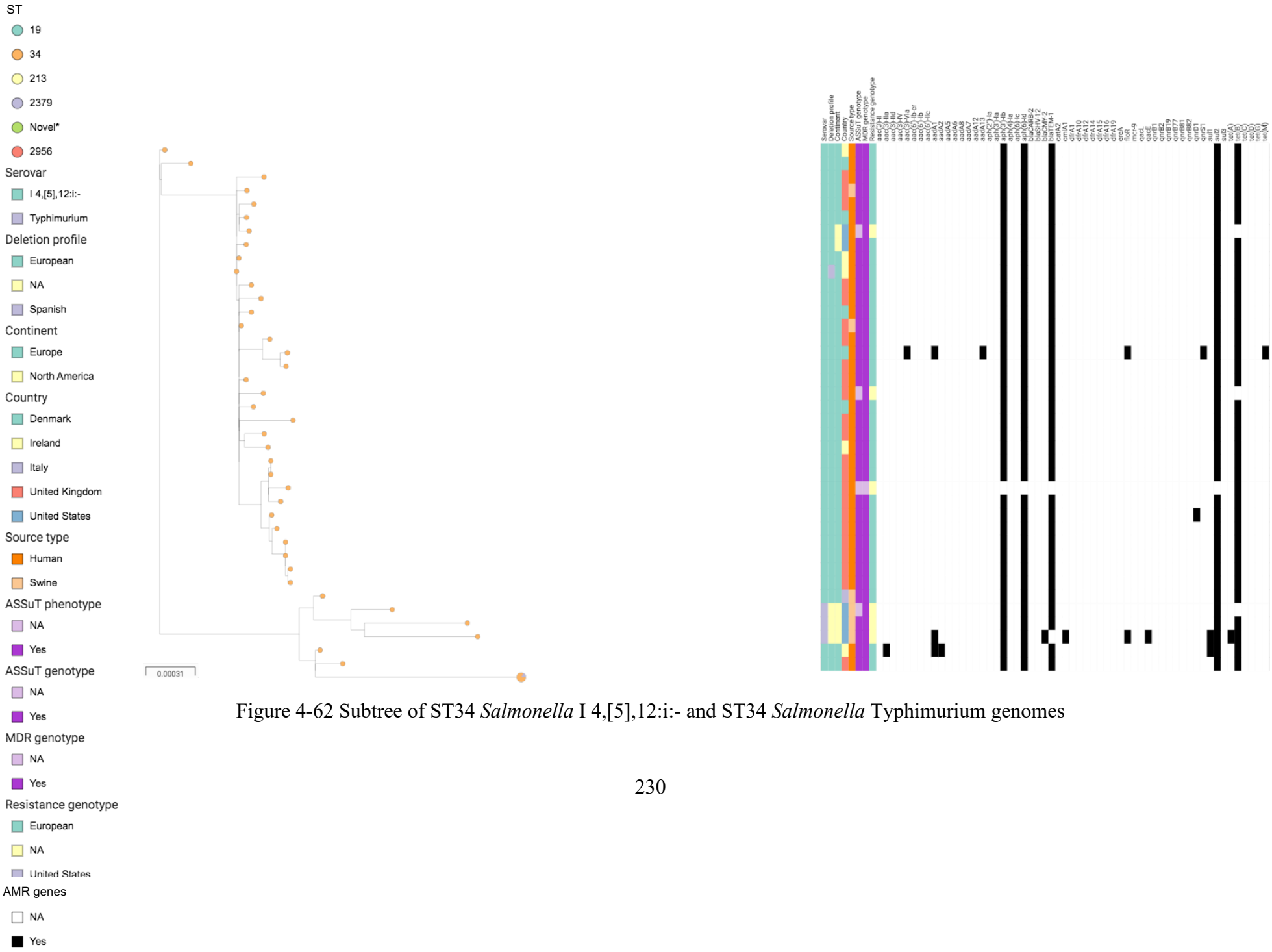


Figure 4-62 Subtree of ST34 *Salmonella* I 4,[5],12:i:- and ST34 *Salmonella* Typhimurium genomes

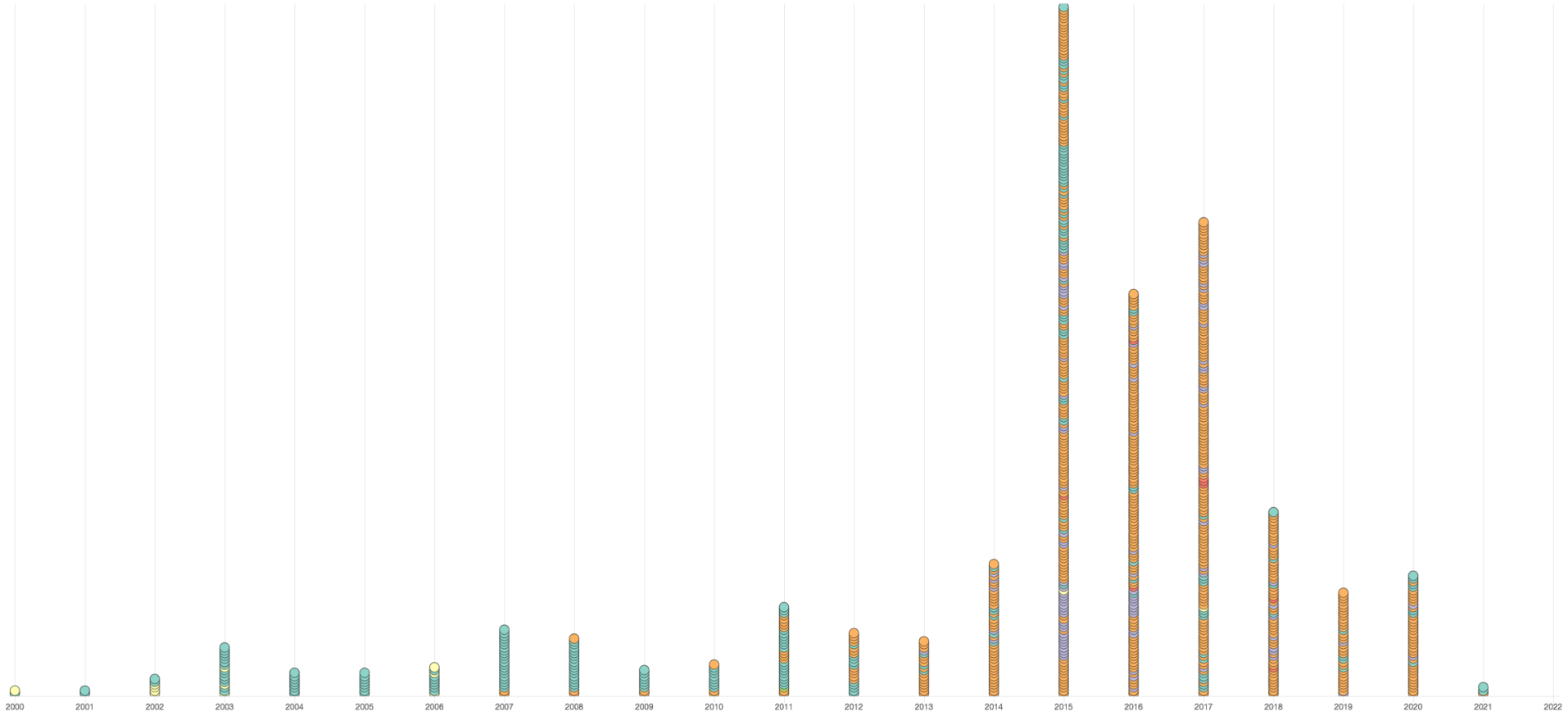


Figure 4-63 Timeline of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolation based on Enterobase metadata

5. DISCUSSION

The objectives of this study were to identify key phenotypic and genotypic characteristics of *Salmonella* I 4,[5],12:i:- that allowed this serovar to emerge and establish an ecological niche in swine production and pork processing. *Salmonella* I 4,[5],12:i:- are commonly isolated from swine, particularly at slaughter; therefore, there is a public health risk as contaminated pork and pork products can enter the food chain and lead to salmonellosis in humans. The phenotypic traits that may be related to increased fitness in swine and their environment such as antimicrobial resistance profiles, fitness, motility, and biofilm production of *Salmonella* I 4,[5],12:i:- were compared to *Salmonella* Typhimurium and other monophasic *Salmonella* serovars. Additionally, genotypic characteristics (MLST, AMR genes, HMT genes and biofilm-associated genes) of *Salmonella* I 4,[5],12:i:- were compared to determine traits that may be associated with a selective and competitive advantage over other *Salmonella* serovars, particularly *Salmonella* Typhimurium. Most importantly, phylogenetic analyses were performed on *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes from swine and humans to assess genetic relatedness. Although several studies have established that *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium are genetically and antigenically similar, few studies have investigated whether there are specific characteristics that are advantageous (or disadvantageous) to *Salmonella* I 4,[5],12:i:-, particularly regarding the lacking the phase 2 flagellar antigen.

As the incidence in humans and prevalence in swine of *Salmonella* I 4,[5],12:i:- continues to increase in the United States and worldwide, our collection of *Salmonella* I 4,[5],12:i:- strains provides a unique prospective as they were isolated from head trim and cheek meat from carcasses of healthy swine in the United States. Additionally, sampling took place at one of the largest commercial pork-processing plants in the southern United States, which serviced numerous grower farms in the Midwest. Thus, the *Salmonella* isolates in our study were representative of *Salmonella* in market hogs from Midwestern United States farms. Furthermore, the isolation of *Salmonella* strains from market-ready hogs which have been thoroughly processed and identified as safe for human consumption provided further insight to the pathogenicity of successful *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium as well as other serovars. This allowed for comparison of *Salmonella* strains from the same sample population. Additionally, this is the first reported study to compare and evaluate phenotypic characteristics of monophasic *Salmonella* strains to *Salmonella* I 4,[5],12:i:- to determine if the expression of only one flagellar antigen gene affects traits related to pathogenicity.

5.1. Evaluation of antimicrobial resistance patterns and heavy metal tolerance genes

Salmonella I 4,[5],12:i:- had four different phenotypic resistance profiles (all MDR) whereas *Salmonella* Typhimurium isolates had 10 different phenotypic resistance profiles (ranging from pan-susceptible to MDR). *Salmonella* I 4,[5],12:i:- (98%) primarily harbored the common resistance genotype $bla_{\text{TEM-1,}}$ *str*A-*str*B, *sul2*, and *tet*(B) showing

phenotypic resistance to ASSuT (one did not show ampicillin resistance (SSuT) despite harboring *bla*$_{\text{TEM-1}}$). Moreover, 76% of all *Salmonella* I 4,[5],12:i:- also harbored a plasmid-mediated quinolone resistance gene (*qnrB*) with most harboring *qnrB*19 and only 3 harboring *qnrB*2. Those with *qnrB*2 also harbored the *bla*$_{\text{SHV-12}}$ gene. In addition to exhibiting the ASSuT resistance genotype, all our *Salmonella* I 4,[5],12:i:- isolates were ST34. Based on several studies that have identified the common resistance phenotypes and genotypes of three *Salmonella* I 4,[5],12:i:- clones, our results indicate that the swine-derived *Salmonella* I 4,[5],12:i:- isolates in our collection identify as the European clone [21, 72, 79].

While most *Salmonella* Typhimurium were also MDR, SSuT (~29%) and ASSuT (~42%), the genotypic resistance patterns differed to MDR *Salmonella* I 4,[5],12:i:-. In contrast to *Salmonella* I 4,[5],12:i:-, ampicillin, streptomycin-spectinomycin, sulfisoxazole, and tetracycline resistance were primarily encoded by *bla*$_{\text{CARB-2}}$, *aadA* and/or *strA-strB*, *sul1*, and *tet*(A) or *tet*(G) genes in *Salmonella* Typhimurium, respectively. Additionally, ~29% *Salmonella* Typhimurium also harbored *floR* encoding resistance to chloramphenicol presenting the ACSSuT resistance profile. The ACSSuT is known to be a historically important MDR pattern mainly in *Salmonella* Typhimurium [263]. Similarly to *Salmonella* I 4,[5],12:i:-, few *Salmonella* Typhimurium (n = 4) harbored both the *qnrB*2 and *bla*$_{\text{SHV-12}}$ genes. ASSuT resistant *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium harboring *qnrB*2 and *bla*$_{\text{SHV-12}}$ shared common AMR genes

encoding resistance to streptomycin-spectinomycin (*aad*A2 and *strA-strB*), aminoglycosides (*aph(3')-Ia*), ampicillin ($bla_{\text{TEM-1}}$), trimethoprim/sulfamethoxazole (*dfrA19*), polymyxins (*mcr*-9), sulfonamides (*sul1* and *sul2*), and tetracyclines (*tet*(D)). Therefore, these strains are resistant to five antibiotic classes. The similar resistance genotype suggests the transfer of plasmids carrying multiple AMR genes may be currently circulating in swine farms and abattoirs in the United States Midwest. Similarly, a previous study identified clinical *Salmonella* I 4,[5],12:i:- as the European clone by the sequence type and genotypic resistance pattern. The VDL *Salmonella* I 4,[5],12:i:- were isolated from swine in 2015 by the Minnesota Veterinary Diagnostic Laboratory (VDL) from farms the United States Midwest [264]. Overall, *Salmonella* Typhimurium had more diverse phenotypic and genotypic profiles than *Salmonella* I 4,[5],12:i:- and showed resistance to additional antibiotics such as amoxicillin/clavulanic acid, cefoxitin, ceftiofur, and chloramphenicol.

Salmonella I 4,[5],12:i:- with *qnrB*19 showed decreased susceptibility to ciprofloxacin with a MIC of 0.5 µg/ml (except one) and most were intermediate to enrofloxacin with an a MIC of 2 µg/ml. Twenty-eight of these isolates showed resistance to nalidixic acid which is often correlated with non-susceptibility to ciprofloxacin and may predict fluroquinolone treatment failure [120, 265]. On the other hand, *Salmonella* I 4,[5],12:i:- with *qnrB*2 showed decreased susceptibility to ciprofloxacin with a MIC of 0.25 µg/ml and were not resistant to nalidixic acid. The MIC of *Salmonella* I 4,[5],12:i:-

with *qnrB*2 also differed for enrofloxacin, where two of the three were intermediate to enrofloxacin with a MIC of 1 μg/ml and one was susceptible. This reveals differences in susceptibility to fluoroquinolones based on the *qnrB* variant present in *Salmonella*. The *Salmonella* I 4,[5],12:i:- *qnrB*19 variant results in a higher degree of decreased the susceptibility to both ciprofloxacin and enrofloxacin greatly compared to the *qnrB*2 variant in *Salmonella* I 4,[5],12:i:-. These results are a cause for concern as *Salmonella* I 4,[5],12:i:- with decreased susceptibility or resistance to enrofloxacin may also indicate decreased susceptibility to the structurally similar fluoroquinolone, ciprofloxacin. which may lead to treatment failure in severe *Salmonella* infections in humans. Furthermore, although nalidixic acid is not used to treat invasive salmonellosis, monitoring susceptibility to this antimicrobial is important for surveillance. Previous studies have also observed *Salmonella* I 4,[5],12:i:- with PMQR genes in clinical livestock as well as chromosomal point mutations in genes encoding quinolone target enzymes [35, 78, 183]. *Salmonella* Typhimurium harboring *qnrB*2 showed similar phenotypic resistance to ciprofloxacin (MIC 0.25 μg/ml) and enrofloxacin (MIC 1 μg/ml) as *Salmonella* I 4,[5],12:i:- also harboring *qnrB*2. The presence of PMQR genes was more prevalent in *Salmonella* I 4,[5],12:i:- than *Salmonella* Typhimurium isolated from swine, indicating *Salmonella* I 4,[5],12:i:- harboring PMQR genes may have a competitive advantage over *Salmonella* Typhimurium under the enrofloxacin selection pressure imposed during the treatment of swine. Furthermore, *Salmonella* I 4,[5],12:i:- with *qnrB*19 are less susceptible

to fluoroquinolones compared to *Salmonella* Typhimurium and I 4,[5],12:i:- with *qnrB*2 suggesting *qnrB*19 provides a greater benefit than *qnrB*2. Importantly, our *Salmonella* I 4,[5],12:i:- isolates contribute to the rise of reduced susceptibility or resistance to fluoroquinolones reported in the United States [35].

All *Salmonella* I 4,[5],12:i:- harboring the $bla_{\text{SHV-12}}$ gene were intermediate to ceftiofur (MIC 4 µg/ml) and resistant to ceftriaxone with two having a MIC of 4 µg/ml and one with a MIC of 1 µg/ml. On the other hand, 3 *Salmonella* Typhimurium harboring the same $bla_{\text{SHV-12}}$ gene were resistant to ceftiofur (8 µg/ml) while one was intermediate. All *Salmonella* Typhimurium were ceftriaxone resistant with MICs ranging from 4 to 16 µg/ml. The $bla_{\text{SHV-12}}$ is an ESBL gene that confers resistance to penicillin, monobactams, and newer cephalosporin generations such as ceftiofur and enrofloxacin [135]. Based on the 2015 NARMS Now: Integrated Report, *Salmonella* I 4,[5],12:i:- harboring $bla_{\text{SHV-12}}$ is known to be infrequent and has only been reported in six *Salmonella* I 4,[5],12:i:- isolates, three of which were in market hogs [140]. In previous studies, $bla_{\text{SHV-12}}$ has been frequently associated with *E. coli* in pigs [266, 267]. Similarly to *Salmonella* I 4,[5],12:i:- harboring $bla_{\text{SHV-12}}$ in our study, there were certain *E. coli* with $bla_{\text{SHV-12}}$ of swine origin that did not show resistance to ceftiofur compared to ceftiofur-resistant *E. coli* with $bla_{\text{CMY-2}}$. Our findings shows that *Salmonella* I 4,[5],12:i:- with $bla_{\text{SHV-12}}$ are less resistant to ceftiofur than *Salmonella* Typhimurium with $bla_{\text{SHV-12}}$, however both were still resistant

to ceftriaxone, the antibiotic used for treatment of in human *Salmonella* infections in humans.

In our study, we found all *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium showed presence of chromosomal copper tolerance genes (*bhsA, comR, copA, cuePR, cutACER*). All *Salmonella* I 4,[5],12:i:- harbored plasmid-borne copper tolerance genes (*pcoABCDRS*) compared to ~26 of *Salmonella* Typhimurium isolates (*pcoABCDR* or *pcoS*). Copper (and zinc) are essential components for cellular metabolic pathways in bacteria and are required for bacterial growth; however, copper is also known to be bactericidal [268]. Therefore, copper toxicity necessitates the need for mechanisms to eliminate excess copper. Essential mechanisms are often encoded by the chromosomal copper tolerance genes mentioned above. Mechanisms include oxidation of copper, regulatory systems (regulators, repressors, or two-component systems to expression of sequestration and active extrusion), copper-binding proteins (copper chaperones), preservation of CPx/P1-type ATPases, and the ancillary copper-efflux system [149, 269]. However, the use of copper in swine as an antimicrobial alternative and/or growth promotion exacerbates the levels of toxicity within the cell providing the need for additional mechanisms that mediate copper resistance such as plasmid-mediated copper tolerance genes (e.g. PCO operon) [77, 111, 270]. All *Salmonella* I 4,[5],12:i:- harbored plasmid-borne copper (*pcoABCDRS*), mercury (*merACDEPT* and *merR1*), and silver (*silABCEFPS*) tolerance genes, while the presence of heavy metal tolerance genes varied

across *Salmonella* Typhimurium with only a few harboring copper (*pcoABCDR* or *pcoS*), mercury (*merACDEPRT, merADEPRT* and *mer*R2 or *merT* and *mer*R2), and/or silver (*silABCEFPS*) tolerance genes. Similarly, a study by Eleonora Mastrorilli et al. showed that the majority of their *Salmonella* I 4,[5],12:i:- (86%) isolates from Italy had copper (*pcoABCDE, cusR*, *cusB, cusA, cusF, cusF,* and *cusC*) and silver (*silADCE*) metal tolerance genes, while 54% of *Salmonella* I 4,[5],12:i:- isolates also contained mercury (*merACPRT*) resistance genes [114]. Petrovska et al. first identified a novel genomic island (SGI-3) specific to *Salmonella* I 4,[5],12:i:- and encoding tolerance to heavy metals such as copper and zinc [14]. Our study demonstrates a high prevalence of heavy metal tolerance genes in MDR *Salmonella* I 4,[5],12:i:-, which may be attributed to the use of heavy metals as supplements in swine feed as micronutrients and antibiotic alternatives.

There is a clear distinction between the genotypic and phenotypic profiles of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates in our study. All ST34 *Salmonella* I 4,[5],12:i:- harbored similar multidrug-resistant resistance genes and heavy metal tolerance genes (copper, silver, and mercury), while ST19 *Salmonella* Typhimurium antimicrobial resistance and heavy metal tolerance genes varied greatly. *Salmonella* I 4,[5],12:i:- were commonly resistant to ampicillin, streptomycin, sulfonamides, and tetracycline (ASSuT). Most also harbored the plasmid-mediated quinolone resistance gene *qnrB* (predominantly *qnrB*19), and as a result were resistant to nalidixic acid and showed decreased susceptibility to fluoroquinolones like ciprofloxacin and enrofloxacin. A small

number of isolates were also resistant to ceftriaxone and trimethoprim/sulfamethoxazole. The NARMS Human Isolates Surveillance report for 2015 found *Salmonella* I 4,[5],12:i:- to be the most common *Salmonella* serovar with ASSuT resistance, accounting for 74.6% of *Salmonella* isolates with the ASSuT resistance pattern collected in 2015. Furthermore, *Salmonella* I 4,[5],12:i:- (67.8%) was the second most common serovar with multidrug-resistance (resistance to three or more classes) followed by *Salmonella* Typhimurium (18.3%) which had been decreasing over time (2004 – 2008) [28]. Our study further demonstrates that *Salmonella* I 4,[5],12:i:- strains isolated from swine in the United States, are contributing to the rise in MDR *Salmonella* as stated by the NARMS Integrated Report, 2015.

Antimicrobial and heavy metal use in swine production provide a selection pressure for *Salmonella* leading to the selection and transmission of AMR and HMT genes. Often, co-selection of AMR and HMT genes occurs as they are co-located on the same mobile genetic element [116, 117]. Furthermore, enrofloxacin and ceftiofur use in swine are known to have important implications for human health as they belong to the same antibiotic class as the human antibiotics ciprofloxacin (fluoroquinolone) and ceftriaxone (third-generation cephalosporin). Ciprofloxacin and ceftriaxone are used to treat severe and invasive *Salmonella* infections in humans. Importantly, fluoroquinolones and cephalosporins antimicrobials are classified as “critically important highest priority” by the World Health Organization [60]. The decreased susceptibility to ciprofloxacin in

most *Salmonella* I 4,[5],12:i:- and resistance to ceftriaxone may lead to delayed response and/or treatment failure. Our study demonstrates *Salmonella* I 4,[5],12:i:- isolated from swine carcasses are multidrug resistant and heavy metal tolerant providing a potential competitive advantage to *Salmonella* I 4,[5],12:i:- in swine and their environment.

5.2. Evaluation of the fitness cost of important antimicrobial resistance genes under selection pressures common in the swine industry

In swine production, antimicrobials such as enrofloxacin, ceftiofur, and tetracycline are frequently used to prevent, treat, and control certain diseases, particularly respiratory diseases. The use of such antimicrobials acts as a selection pressure, which often results in acquisition of antimicrobial resistance genes or chromosomal mutations in intestinal and extra-intestinal bacteria, including *Salmonella*. This allows resistant bacteria, such as multidrug-resistant *Salmonella* I 4,[5],12:i:-, to survive and replicate in swine in the presence of the antimicrobial [117]. Antimicrobial resistance is frequently associated with a fitness cost that is typically seen as a reduction in bacterial growth rate [161, 162]. The biological cost of resistance is an important factor that influences the rate of resistance gene transfer/mutations, rate of decreased resistance if antimicrobial use were reduced, and the stability of resistance [162]. As mentioned, the majority of the *Salmonella* I 4,[5],12:i:- strains in our study were MDR harboring resistance genes to ampicillin ($bla_{\text{TEM-1}}$), streptomycin (*strA-B*), sulfonamides (*sul2* and *sul1*), and tetracycline (*tet*(B) and *tet*(D)). Additionally, many strains harbored a plasmid quinolone resistance gene

(*qnrB*19 or *qnrB*2), and a few others also harbored an extended spectrum beta-lactamase resistance gene (*bla*$_{\text{SHV-12}}$). One of the fundamental factors in determining the evolutionary success of *Salmonella* I 4,[5],12:i:- in swine, is determining its relative fitness in the presence and absence of antimicrobials frequently administered in the swine industry. Our study is the first to evaluate the fitness costs *in vitro* of antimicrobial resistance genes found in *Salmonella* 4,[5],12:i:- and other *Salmonella* serovars under the selection of antimicrobials such as enrofloxacin, ceftiofur, and tetracycline. Furthermore, our collection of *Salmonella* strains provide insight to the relative fitness and biological cost of resistance genes of successful *Salmonella* isolated from ready-to-market swine carcasses.

In our study, there was no fitness cost to harboring *qnrB*19 for *Salmonella* I 4,[5],12:i:-. However, as enrofloxacin concentrations increased, the relative fitness of *Salmonella* I 4,[5],12:i:- with *qnrB*19 decreased. Thus, the relative fitness of *Salmonella* I 4,[5],12:i:- with *qnrB*19 was greater at lower concentrations of enrofloxacin than at higher concentrations. As expected, growth was inhibited at 4 µg/ml (resistance for *Salmonella*) as the majority of *Salmonella* I 4,[5],12:i:- harboring *qnrB*19 had a MIC of 2 µg/ml, while a few had a MIC of 1 µg/ml. Plasmid-mediated quinolone resistance (PMQR) genes such as *qnrB* provide reduced susceptibility to fluoroquinolones and not resistance. Clinical resistance to fluoroquinolones/quinolones requires more than one mechanism of resistance

such as chromosomal point mutations in the target enzymes (e.g. *gyrA* and *parC*), active efflux, and/or acquisition of PMQR genes.

A unique growth curve was observed at the enrofloxacin concentration of 2 µg/ml. Before 1000 minutes, growth was nonexistent and similar to the growth at 4 µg/ml, but after 1000 minutes, the growth rate increased exponentially and reached the stationary phase (Figure 4-3). Although the length of the lag phase was longer than at the lower concentrations of enrofloxacin, the eventual exponential growth indicates that *Salmonella* I 4,[5],12:i:- with *qnrB*19 was able to adjust to the 2 µg/ml enrofloxacin environment, and replicate. This was not surprising, given that many of the isolates with *qnrB*19 had a MIC of 2 µg/ml. The adjustment may be due to the theory of stepwise resistance. Stepwise resistance is defined as the stepwise selection of successive or compensatory mutations at non-lethal or sub-lethal selection pressures, such as antimicrobials. Because acquisition of resistance genes may come at a fitness cost, the cost may be reduced or compensated by the presence of such mutation. At nonlethal antimicrobial concentrations (≤MIC), resistant mutants with low-level resistance to antimicrobials (e.g. fluoroquinolones) are known to emerge [116, 129, 271-273]. However, combined with another resistance mechanism attained through compensatory mutation, high-level resistance may occur [162, 164]. Therefore, *Salmonella* I 4,[5],12:i:- with *qnrB*19 may have developed a compensatory mutation (e.g. DNA gyrase, DNA topoisomerase IV, or regulators of efflux pumps) at 2 µg/ml, allowing for increased fitness (increase in growth rate) without the loss of the

original level of resistance. Several studies have shown a significant reduction in bacterial fitness in *E. coli* with single fluoroquinolone resistance mutations (both *in vitro* and *in vivo*) which have led to the selection of compensatory mutations [165, 274, 275]. One particular study by Huseby et al. (2017) showed that fitness decreased with the number of fluoroquinolone resistance mutations; however, triple-mutants obtaining a fourth fluoroquinolone resistance mutation (usually *parC*) increased in fitness and fluoroquinolone resistance considerably [165]. For future studies, it would be interesting to see what compensatory mutation, if any, were attained by these *Salmonella* I 4,[5],12:i:- isolates with *qnrB*19.

Similar to *Salmonella* I 4,[5],12:i:- with *qnrB*19, *Salmonella* serovars Derby and *Salmonella* Senftenberg harboring the *qnrB*19 gene showed no fitness cost. *Salmonella* serovars Derby and *Salmonella* Senftenberg also had a *parC* mutation. In contrast to *Salmonella* I 4,[5],12:i:- with only the *qnrB*19, both serovars showed increased fitness when no antibiotic was present and a greater fitness at 0.25 µg/ml. Additionally, the combination of both resistance genes (*qnrB*19 and *parC*) allowed for growth at higher concentrations of enrofloxacin (4 µg/ml) well above the MICs of each serovar (0.5 or 1 µg/ml) whereas *Salmonella* I 4,[5],12:i:- with only *qnrB*19 was inhibited. This suggests the additional presence of the fluoroquinolone resistance point mutation in *parC* (encoding DNA topoisomerase IV subunit A) greatly enhances the fitness of *Salmonella* serovars Derby and Senftenberg under no antibiotic pressure and at varying concentrations of

enrofloxacin even with growth reaching the breakpoint for enrofloxacin (4 µg/ml). Aforementioned, similar findings were observed in a study by Huseby et al. (2017), where *parC* increased the fitness and level of resistance to fluoroquinolones in *E. coli.* This study also found *parC* point mutations to occur more frequently than high-cost efflux regulator mutations, likely due to having a minor fitness cost [274]. Thus, fluoroquinolone use in swine may result in the stepwise selection of successive mutations at non-lethal concentrations by enriching high-fitness *Salmonella* with reduced susceptibility (PMQR genes).

In contrast to *Salmonella* I 4,[5],12:i:- isolates with the *qnrB*19 gene, *Salmonella* I 4,[5],12:i:- harboring *qnrB*2 were less fit at lower concentrations of enrofloxacin and growth was inhibited at 1 µg/ml rather than 2 µg/ml, as seen in isolates with *qnrB*19. Although, there was also no fitness cost to harboring *qnrB*2, our results indicate *Salmonella* I 4,[5],12:i:- with *qnrB*19 have greater fitness and provide greater reduced susceptibility to enrofloxacin than *qnrB*2. Although *Salmonella* serovars I 4,[5],12:i:- and Typhimurium harbored the *qnrB*2 gene and expressed similar MICs for enrofloxacin (1 µg/ml), there was a minor fitness cost for *Salmonella* Typhimurium harboring *qnrB*2 and the fitness for each serovar differed across enrofloxacin concentrations. There were two instances where the fitness of *Salmonella* Typhimurium with *qnrB*2 was greater than *Salmonella* I 4,[5],12:i:- with *qnrB*2; (1) at the lowest concentration of enrofloxacin (0.25 µg/ml) and (2) at 1 µg/ml where *Salmonella* I 4,[5],12:i:- growth was inhibited while

Salmonella Typhimurium was inhibited at 2 µg/ml. The growth curve at 1 µg/ml for *Salmonella* Typhimurium was similar to the growth curve of *Salmonella* I 4,[5],12:i:- with *qnrB*19 at 2 µg/ml, in that growth was nonexistent and increased exponentially over time reaching the stationary phase indicating that *Salmonella* Typhimurium with *qnrB*2 was able to adapt and grow at the 1 µg/ml enrofloxacin environment. Thus, although *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- harbor the same PMQR gene (*qnrB*2), *qnrB*2 possibly provides a slightly greater fitness advantage to *Salmonella* Typhimurium in enrofloxacin. It is also important to note that the additional resistance genes found in the *Salmonella* Typhimurium and I 4,[5],12:i:- isolates could influence the fitness, even if the antibiotics are not directly providing a selection pressure.

On the other hand, *Salmonella* Senftenberg isolates that harbored *qnrB*2, *aac(6')-Ib-cr*, and a *parC* point mutation showed a slight fitness cost at the stationary phase with no antibiotic present which may be attributed to *qnrB*2 as seen in *Salmonella* Typhimurium isolates harboring *qnrB*2 or the presence of *aac(6')-Ib-cr*. Unfortunately, we were unable to determine which particular gene resulted in reduced fitness as there were no *Salmonella* isolates solely harboring one particular resistance gene. Though, we did observe that the combination of these genes increased the fitness of *Salmonella* Senftenberg at low concentrations of enrofloxacin compared to no antibiotic being present and allowed growth at 1 and 2 µg/ml which is beyond the MIC of these strains (0.5 µg/ml). These results show that the presence of three fluoroquinolone resistance mechanisms

(*qnrB*2, *aac(6')-Ib-cr*, and *parC* mutation) enhances fitness in enrofloxacin, similar to the results observed in the previously mentioned studies [165-167]. However, *Salmonella* Senftenberg with *qnrB*19 and a *parC* mutation showed resistance to enrofloxacin with growth at 4 μg/ml suggesting *qnrB*19 and the *parC* mutation provides a greater fitness advantage than those with *qnrB*2, *aac(6')-Ib-cr*, and *parC* mutation. The difference in fitness may be attributed to the presence of *qnrB*19. Similar results were also seen in with *Salmonella* I 4,[5],12:i:- harboring *qnrB*19 which had a greater fitness than *Salmonella* I 4,[5],12:i:- harboring *qnrB*2. However, *Salmonella* Senftenberg with either *qnrB* gene (*qnrB*19 or *qnrB*2) and a *parC* mutation, had greater growth rates than *Salmonella* I 4,[5],12:i:- with either *qnrB* gene (*qnrB*19 or *qnrB*2), indicating the presence of additional resistance mechanisms further enhances the fitness of *Salmonella* strains. Overall, this suggests that when swine are treated with enrofloxacin, *Salmonella* with fluoroquinolone resistance genes encoding reduced susceptibility still have the ability to grow under the selection pressure of the antibiotic and may survive and even replicate within the host.

About 78% of the *Salmonella* I 4,[5],12:i:- that were isolated from swine tissue samples from the current study harbored a *qnrB* (*qnrB*2 or *qnrB*19) resistance gene. Enrofloxacin administered to swine may contribute to the selection of fluoroquinolone resistant (or reduced susceptibility) *Salmonella* I 4,[5],12:i:- strains with increased fitness. There was no fitness cost to harboring either plasmid-mediated quinolone resistance gene, (*qnrB*2 or *qnrB*19), and each gene provided different phenotypic profiles with *qnrB*19

isolates having a greater MIC and fitness than those with *qnrB*2. The reduction in susceptibility to enrofloxacin may reflect decreased susceptibilty to ciprofloxacin, which can complicate treatment of invasive *Salmonella* infections. Ciprofloxacin is an antibiotic in the same class as enrofloxacin and is used to treat severe *Salmonella* infections in humans [120, 276]. Delsol et al. showed that enrofloxacin treatment in pigs selected for quinolone-resistant *Salmonella* Typhimurium DT104 strains suggesting a competitive advantage over susceptible *Salmonella* Typhimurium DT104 strains [118]. Although there are no studies on the effect of enrofloxacin use in pigs on *Salmonella* I 4,[5],12:i:-, our study highlights the fact that *Salmonella* I 4,[5],12:i:- with reduced susceptibility may also have a competitive advantage in the swine industry where enrofloxacin treatment is commonly used.

Ceftiofur, a third-generation cephalosporin, is another commonly used antimicrobial in swine and resistance is often encoded by extended spectrum beta-lactam (ESBL) genes such as *bla*$_{\text{SHV-12}}$. In our study, we assessed the fitness of *Salmonella* I 4,[5],12:i:- and other *Salmonella* serovars harboring *bla*$_{\text{SHV-12}}$ grown at different concentrations of ceftiofur. The growth rates of *Salmonella* serovars I 4,[5],12:i:-, Typhimurium, and Senftenberg isolates harboring *bla*$_{\text{SHV-12}}$ decreased as the concentrations of ceftiofur increased. However, growth still occurred at higher concentrations of ceftiofur (6 and/or 8 μg/ml) and an increase in fitness was observed at the stationary phase compared to their baseline (no antibiotic). When comparing

Salmonella serovars Typhimurium and I 4,[5],12:i:- with *bla*$_{SHV-12}$, a fitness cost was observed in *Salmonella* Typhimurium strains at all growth phases while *Salmonella* I 4,[5],12:i:- had no fitness cost to harboring *bla*$_{SHV-12}$. *Salmonella* Senftenberg also showed a slight fitness cost to harboring the *bla*$_{SHV-12}$ gene; however, only at the stationary phase. The presence of *bla*$_{SHV-12}$ appears to be beneficial to *Salmonella* I 4,[5],12:i:- and *Salmonella* Senftenberg as there was a slight increase in fitness under no antibiotic pressure and at 2 μg/ml of ceftiofur, respectively. *Salmonella* I 4,[5],12:i:- were also more fit than *Salmonella* Senftenberg and Typhimurium at the stationary phase across all concentrations. Although the *Salmonella* serovars I 4,[5],12:i:-, Typhimurium, and Senftenberg harbored the same ESBL gene, *bla*$_{SHV-12}$, the relative fitness differed at each growth phase and not all serovars had a fitness cost. Previous studies have shown plasmids carrying antimicrobial resistance genes typically impose a fitness cost on the bacterial hosts that harbor them [277-279]. Dahlberg et al. (2003), observed plasmids R1 and RP4 harboring MDR genes imposed a fitness cost on *E. coli*, which was determined in competition experiments. However, through experimental evolution, they also determined the costs associated with plasmid carriage can be compensated by mutations on the plasmid itself or the chromosome [277]. Therefore, the differences in fitness costs may be attributed to the plasmids carrying the *bla*$_{SHV-12}$ gene and the more fit *Salmonella* serovars harboring *bla*$_{SHV-12}$ may be a result of genetic changes in the chromosome or plasmid that reduced that cost.

Although, there are currently no fitness studies of *Salmonella* regarding the *bla*$_{SHV-12}$ gene, Ogunrinu et al. (2020) investigated the fitness cost of *E. coli* with different beta-lactamase genes. They found no fitness cost to harboring variants of the *bla*$_{CTX-M}$ gene, a gene also classified as an extended-spectrum beta-lactamase (ESBL) like *bla*$_{SHV-12}$. Similarly in our study, *Salmonella* I 4,[5],12:i:- showed no fitness cost to harboring *bla*$_{SHV-12}$. As an ESBL gene, *bla*$_{SHV-12}$ is known to provide resistance to third generation cephalosporins such as ceftiofur (resistance MIC $\geq$ 8 μg/ml) and ceftriaxone (resistance MIC $\geq$ 4 μg/ml). Interestingly, *bla*$_{SHV-12}$ provided phenotypic resistance to ceftiofur for three out of four *Salmonella* Typhimurium and six out of 7 *Salmonella* Senftenberg, whereas all three *Salmonella* I 4,[5],12:i:- strains harboring *bla*$_{SHV-12}$ gene were classified as intermediate. The fitness cost to harboring *bla*$_{SHV-12}$ in *Salmonella* serovars Typhimurium and Senftenberg may be due to encoding a greater level of resistance in these serovars compared to *Salmonella* I 4,[5],12:i:-. Additionally, *Salmonella* serovars Typhimurium and Senftenberg had shorter lag phases and greater exponential growth rates across all concentrations likely due to the higher MIC values. However, growth rates for *Salmonella* I 4,[5],12:i:- were greater than *Salmonella* serovars Typhimurium and Senftenberg at all phases and concentrations of ceftiofur. The continued growth at higher concentrations of ceftiofur suggests *bla*$_{SHV-12}$ increased the fitness of *Salmonella* I 4,[5],12:i:-.

While *Salmonella* I 4,[5],12:i:- with *bla*$_{SHV-12}$ were not phenotypically resistant to ceftiofur, all were resistant to ceftriaxone (MICs of 4 µg/ml and 8 µg/ml), another third-generation cephalosporin that is important in human health. Ceftriaxone is used to treat severe cases of salmonellosis in humans [133, 280]. Interestingly, *Salmonella* Typhimurium and *Salmonella* Senftenberg with *bla*$_{SHV-12}$ also showed resistance to ceftriaxone (varying MICs of 4-32 µg/ml). Future studies should include determining the fitness cost of *bla*$_{SHV-12}$ under the selection pressure of ceftriaxone to determine relevance to resistance and fitness of clinically resistant strains. This is important because ceftriaxone and ciprofloxacin are often administered for severe cases of salmonellosis in humans.

Two experimental studies assessing the selection pressure of ceftiofur use on *E. coli* and *Salmonella* in pigs and cattle, respectively, showed an increase in MDR bacterial populations right after treatment followed by decreasing MDR populations over time [127, 130]. The fitness cost of ceftiofur resistance genes like *bla*$_{SHV-12}$ may play a role in the decrease of the *Salmonella* resistant population. After treatment, the volume/concentration of ceftiofur in the animal is reduced over time resulting in more fit susceptible strains outcompeting the less fit resistant strains. However, the fact that *Salmonella* I 4,[5],12:i:- with *bla*$_{SHV-12}$ showed no fitness cost, had increased fitness, and were intermediate rather than resistant to ceftiofur suggests *Salmonella* I 4,[5],12:i:- with *bla*$_{SHV-12}$ may have an advantage in the swine host over other less fit *Salmonella* serovars harboring *bla*$_{SHV-12}$.

Lastly, tetracycline antibiotics such as chlortetracycline and oxytetracycline are widely used in veterinary medicine for their broad-spectrum properties. Their extensive use has led to an increased incidence of tetracycline resistance in *Salmonella* of human and animal origins, including swine [143, 281, 282]. In our study, most *Salmonella* strains (80%) harbored a tetracycline resistance gene (alone or in combination with another *tet* gene). All *tet* genes (*tet*(A), *tet*(B), *tet*(D), and *tet*(G)) identified provided phenotypic resistance to tetracycline (MIC 32 μg/ml) for the *Salmonella* serovars (I 4,[5],12:i:-, Agona, Alachua, Derby, Senftenberg, and Typhimurium) found in this study. The relative fitness of *Salmonella* I 4,[5],12:i:- and Typhimurium and other serovars harboring *tet* genes was evaluated to determine differences between *Salmonella* harboring the same, different, or a combination of *tet* genes.

Interestingly, our study found that monophasic serovars showed no fitness cost to harboring any of the *tet* genes or combination of *tet* genes: (1) *Salmonella* serovars Agona, Derby, Senftenberg harboring *tet*(A), (2) *Salmonella* serovars I 4,[5],12:i:-, Agona, Derby harboring *tet*(B), (3) *Salmonella* Senftenberg harboring *tet*(D), and (4) *Salmonella* Alachua harboring *tet*(A)/*tet*(B) and *Salmonella* I 4,[5],12:i:- harboring *tet*(B)/*tet*(D). On the other hand, *Salmonella* Typhimurium strains harboring *tet*(A), *tet*(A)/*tet*(B), or *tet*(D) showed a fitness cost at the stationary phase. *Salmonella* Typhimurium was the only serovar harboring *tet*(G), and no fitness cost was associated with harboring the *tet*(G) gene. Additionally, *Salmonella* Typhimurium with *tet*(G) had the greatest fitness at all phases

and concentrations of tetracycline than all monophasic serovars regardless of the *tet* gene(s) present. This suggests that when the selective pressure of tetracycline is reduced or absent, monophasic *Salmonella* serovars harboring *tet* gene(s) are more fit than *Salmonella* Typhimurium with the same gene(s). However, our study does not determine whether the lack of a second phase flagellar antigen gene plays a role in the increased fitness of monophasic strains. The majority of isolates had resistance genes in addition to the *tet* genes and this could also impact the relative fitness of strains.

Furthermore, there were differences in fitness among the serovars harboring the same tetracycline resistance gene or when in harboring two *tet* genes. Most had similar growth rates at the exponential phase, whereas differences were seen at the stationary phase. The additional presence of *tet*(D) increased the fitness of *Salmonella* I 4,[5],12:i:- with *tet*(B) compared to those harboring only *tet*(B) in the absence of tetracycline. *Salmonella* I 4,[5],12:i:- with *tet*(B) also showed greater fitness at the stationary phase than Agona with *tet*(B), Typhimurium with *tet*(A)/*tet*(B), and Typhimurium with only *tet*(A). However, *Salmonella* I 4,[5],12:i:- with only *tet*(B) and those *tet*(B)/*tet*(D) had comparable fitness at all concentrations of tetracycline. Furthermore, the additional presence of *tet*(B) in *Salmonella* Typhimurium with *tet*(A) reduced the fitness at all phases of growth and concentrations of tetracycline. In contrast, *Salmonella* Alachua with *tet*(A) was not affected and no fitness cost was observed in other monophasic serovars harboring only *tet*(B). This indicates *tet*(B) may be associated with a fitness cost only in *Salmonella*

Typhimurium Unlike *Salmonella* I 4,[5],12:i:- with *tet*(B)*/tet*(D), which showed greater fitness than *Salmonella* I 4,[5],12:i:- only with *tet*(B), *Salmonella* Typhimurium had a fitness cost to harboring only *tet*(D) in the absence of tetracycline. The fitness of *Salmonella* Typhimurium was also less than *Salmonella* I 4,[5],12:i:- harboring both *tet*(B)*/tet*(D) and *Salmonella* Senftenberg with *tet*(D). Overall, *Salmonella* I 4,[5],12:i:- harboring *tet* gene(s) have greater fitness than *Salmonella* Typhimurium harboring the same resistance genes providing a better advantage under tetracycline selection pressure.

The fitness of *Salmonella* with *tet* gene(s) did not gradually decrease or increase as the concentration of tetracycline increased. The majority of *Salmonella* harboring *tet* gene(s) varied in fitness at the exponential phase, while the fitness of both monophasic and biphasic strains was relatively similar at the stationary phase. The presence of any of the tetracycline resistance genes in this study provides comparable fitness at all concentrations of tetracycline once growth reaches the stationary phase for the each serovar. This may be an indication that the extensive use and/or misuse of tetracycline in swine over time has led to the selection of tetracycline resistance in *Salmonella* at no biological cost. Tetracycline resistance genes found in our *Salmonella* isolates (*tet*(A), *tet*(B), *tet*(D), and *tet*(G)) encode antimicrobial efflux pumps of different classes (A, B, D, and G). These genes can be localized within mobile genetic elements (e.g. transposons or plasmids) in the *Salmonella* genome or may be chromosomally located, which is the case

for *tet*(B) in *Salmonella* I 4,[5],12:i:-. Thus, our results indicate the location of the *tet* genes do not influence the biological fitness cost.

Studies have shown that the use of chlortetracycline at subtherapeutic levels increases the prevalence and proportion of resistant bacteria in pigs and cattle [130, 141, 142]. A study by Ohta et al. observed an increased proportion of MDR *Salmonella* (including resistance to tetracycline) in chlortetracycline treated cattle. In contrast to the ceftiofur treated group in their previous study, half of the *Salmonella* population in the chlortetracycline treated group remained MDR at day 26 [130]. Although there are many factors involved in the persistence of MDR *Salmonella*, the lack of a biological cost to harboring tetracycline resistance genes in MDR *Salmonella* isolated from swine provides a fitness advantage in the presence and absence of tetracycline resistance. If there is no cost of resistance, the AMR resistant population are equally fit as the susceptible population. This supports the theory other studies have suggested, that in community settings, the biological cost of resistance is a significant factor in successfully displacing AMR populations with susceptible ones [163, 283, 284].

Currently, there are no reported experimental studies on the effect of enrofloxacin, ceftiofur, or tetracycline use in pigs on *Salmonella* I 4,[5],12:i:- and whether resistant *Salmonella* I 4,[5],12:i:- have a competitive advantage over resistant *Salmonella* Typhimurium. Therefore, our investigation provides insight into the potential biological fitness cost of antimicrobial resistance genes commonly found in MDR *Salmonella* I

4,[5],12:i:- strains isolated from swine. Additionally, we were able to determine differences in the relative fitness (*in vitro*) between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium and other monophasic strains with diverse phenotypic and genotypic resistance profiles in the presence and absence of antimicrobial selection pressures. Overall, our study shows the importance of *in vitro* fitness studies, as measuring the fitness of resistance genes associated with antibiotics used in swine is a key determinant of *Salmonella* survival in swine and their environment.

Salmonella I 4,[5],12:i:-, and bacterial pathogens in general, are exposed to a wide variety of selection pressures (e.g. antimicrobials, biocides, environmental factors) that differ in concentrations over time and exist in complex combinations in food production. Consequently, the prediction of resistance evolution and true fitness of *Salmonella* under antimicrobial pressure is difficult to determine outside of a laboratory setting. However, it is well-known that antimicrobials used in food production animals are often circulating in animals (e.g. swine) and the environment in non-lethal concentrations, constantly exposing bacterial populations not intended for treatment. Multidrug-resistant *Salmonella* I 4,[5],12:i:- isolated from swine in our study showed no fitness costs to harboring antimicrobial resistance genes encoding resistance to enrofloxacin (*qnrB*19 and *qnrB*2), ceftiofur ($bla_{\text{TEM-1}}$), and tetracycline (*tet*(B) and *tet*(D)). Aside from obtaining antimicrobial resistance genes that allow for survival in swine production, the administration of antibiotics at non-lethal or sub-lethal concentrations may result in

multidrug-resistance and increased fitness of *Salmonella* I 4,[5],12:i:-. Therefore, resistant *Salmonella* I 4,[5],12:i:- may have a selective advantage in swine and their environment due to the increased probability of being enriched under non-lethal/sub-lethal antimicrobial selection pressures and may even be selected in the absence of the antimicrobial when there are no fitness costs associated with the acquired resistance genes.

5.3. Evaluation of **Salmonella** *I 4,[5],12:i:- virulence factors such as motility and biofilm production*

Motility and biofilm formation are important virulence factors that enhance the ability of bacterial spp. to survive and persist within the host and/or environment [37, 42, 45, 196]. Motility of an organism is facilitated by flagella to move towards or away from attractants or repellants. Studies have shown *Salmonella* flagella play a key role in pathogenicity particularly in the initial stages of infection and biofilm development via motility [37]. The ability to produce biofilms can influence the result of an infection and in turn provide protection from the hosts' immune response as well as resistance to antimicrobial agents [194, 195]. Our study aimed to determine if monophasic and biphasic expression of *Salmonella* serovars effects motility (e.g. swarming and swimming) as well as biofilm production. Additionally, we evaluated the effect of known and previously studied *Salmonella* biofilm-related genes on biofilm production. The overall goal was to understand how the lack of the second phase flagellar antigen effects motility and biofilm

production of *Salmonella* I 4,[5],12:i:-, which may negatively or positively impact its pathogenicity in swine and ability to persist in the environment.

In our study, swimming motility varied more than swarming motility across *Salmonella* serovars (biphasic and monophasic). There were no differences in swarming between biphasic *Salmonella* and most of the monophasic *Salmonella* serovars. Differences were only seen at the 6th hour (first time point) where *Salmonella* Typhimurium had greater swarming ability than *Salmonella* serovars Agona and Senftenberg, and *Salmonella* Agona had a greater swarming ability than *Salmonella* I 4,[5],12:i:-. Contrastingly, more differences were seen in swimming motility between biphasic (*Salmonella* Typhimurium) and monophasic *Salmonella*, as well as between monophasic serovars (*Salmonella* serovars I 4,[5],12:i:-, Agona, Alachua, Derby, Enteritidis, Montevideo, and Senftenberg). Biphasic *Salmonella* Typhimurium had greater swimming motility than the monophasic *Salmonella* serovars studied. For the most part, *Salmonella* Senftenberg had the greatest swimming ability among all monophasic serovars while, *Salmonella* I 4,[5],12:i:- had the weakest swimming ability among monophasic serovars (*Salmonella* serovar Agona, Derby, Enteritidis, Senftenberg) followed by *Salmonella* Alachua.

Our results suggest that the lack of a second flagellar phase antigen may affect the swimming ability of monophasic serovars, while the biphasic expression or presence of two flagellar antigens enhances the swimming ability of *Salmonella* Typhimurium, a

biphasic serovar. Nevertheless, the variation among monophasic serovars in swimming shows there may be other factors involved in the ability of *Salmonella* to swim. Bogomolnaya et al. (2014) discovered 130 mutations involved in modulating swimming and/or swarming motility of *Salmonella* Typhimurium [285]. Interestingly, Bogomolnaya et al. (2014) identified approximately 50 mutants that had reduced swimming motility compared to the wildtype, where swarming motility was not affected; the majority had mutations related to the production or structure of lipopolysaccharides (LPS). This suggests the two different types of motilities are mutually exclusive. Potentially, a microscopic view of swarmer cells versus swimmer cells will provide clues regarding the differing phenotypes. Additional motility studies including other biphasic *Salmonella* serovars are needed to determine if strong swimming motility is serovar dependent or only related to biphasic expression and/or the presence of two flagellar antigen genes.

Kim and Surette (2005) found that all *Salmonella enterica* subspecies I exhibited swimming motility and four did not swarm, while four strains from all seven subspecies of *Salmonella* groups did not swim nor swarm. The inability of *Salmonella* strains to swarm was due to lacking the O-antigen component of the LPS and/or mutations in LPS modification genes [286]. However, the *Salmonella enterica* subspecies I swarm-defective strains swarmed on alternate carbon sources (e.g. *N*-acetylglucosamine or arabinose). This was also observed by Toguchi et al. (2000) where LPS mutants were defective in swarming [287]. These studies concluded that swarming motility is an evolutionary

conserved behavior in *Salmonella enterica* subspecies I, most of which have adapted to the nutrient-rich gastrointestinal environment. Furthermore, glucose is an energy-rich carbon source known to be essential for stimulating active swarming motility in *Salmonella* Typhimurium even in nutrient-rich environments [288]. Thus, glucose supplementation was used in our study to stimulate swarming motility *in vitro*, which is physiologically relevant to the nutrient-rich environment of a host's gastrointestinal tract (e.g. human or swine) [289, 290]. Although we did not have any swarm deficient *Salmonella*, the fact that all *Salmonella* serovars (subspecies I) in our study exhibited swarming behavior and were not significantly different suggests our findings are consistent with previous studies.

This is the first study to investigate the differences in motility between biphasic and monophasic *Salmonella* serovars that have been isolated from swine. As mentioned, there were no differences in swarming motility across all serovars, whereas swimming motility varied. Salmonella Typhimurium displayed greater swimming motility than all monophasic serovars including *Salmonella* I 4,[5],12:i:-. Several studies have determined that in comparison to *Salmonella* Typhimurium, monophasic expression of *Salmonella* I 4,[5],12:i:- does not alter the pathogenicity and virulence in swine [32, 33, 96, 106]. This suggests that though *Salmonella* I 4,[5],12:i:- are weak swimmers compared to *Salmonella* Typhimurium, their virulence and pathogenicity in swine are not impaired due to swimming motility. Thus, swimming motility is not an essential phenotype for *Salmonella*

I 4,[5],12:i:- for cell invasion and colonization of tissue in pigs enabling *Salmonella* I 4,[5],12:i:- to persist in the swine industry and spread between swine and humans through consumption of contaminated pork.

In our study, biofilm production varied across all serovars; however, most serovars were weak biofilm producers. *Salmonella* serovars Agona and Alachua were all weak biofilm producers and the majority of *Salmonella* Derby, I 4,[5],12:i:- and Typhimurium isolates were also weak biofilm producers. On the other hand, *Salmonella* serovars Typhimurium and Derby had a few moderate producers, while *Salmonella* serovars Enteritidis and Montevideo had moderate and strong biofilm producers. There were no specific differences in biofilm production between *Salmonella* Typhimurium, a biphasic *Salmonella* serovar, and all the other monophasic serovars. Our findings are consistent with previous studies which have found variability in biofilm formation among *Salmonella* serovars [194, 203-205]. Moreover, there were significant differences between *Salmonella* Typhimurium which had weak biofilm forming ability and *Salmonella* serovars that were strong biofilm producers. The diversity in biofilm production in *Salmonella* serovars and the fact that *Salmonella* Typhimurium, a biphasic serovar, displayed weak biofilm production, suggests that biofilm production is not dependent on the presence of both flagellar antigens and that the lack of one does not hinder the ability to produce biofilms in monophasic serovars.

A study by Yin et al. evaluated biofilm formation of *Salmonella* serovars isolated from beef processing plants and also found *Salmonella* serovars Typhimurium and Agona to be a weak biofilm producers while *Salmonella* serovars Senftenberg and Derby were stronger biofilm producers under various conditions (e.g. temperatures and pH) [204]. However, *Salmonella* serovars I 4,[5],12:i:-, Alachua, Enteritidis, and Montevideo were not included in the study. Another study by Agarwal et al., similarly showed variation in biofilm production of 69 different *Salmonella* serovars, including reference strains and strains originating from different hosts [205]. Interestingly, similar results were seen for *Salmonella* serovars Enteritidis, Montevideo and Derby; where Enteritidis were moderate and strong biofilm producers, Montevideo were moderate biofilm producers, and Derby were weak biofilm producers. In contrast, Agarwal et al., found *Salmonella* Typhimurium, in particular, had weak, moderate, and strong biofilm producers whereas, only weak and moderate biofilm producers were found in our study. The variation in *Salmonella* Typhimurium biofilm production may be because *Salmonella* Typhimurium has a broad-host range and is the most commonly cultured and characterized *Salmonella* serovar.

Most studies investigating biofilm production in *Salmonella* are based on the well characterized pathogens *Salmonella* Typhi (Typhi) and *Salmonella* Typhimurium (non-Typhi) [43, 44, 193, 291, 292]. Although *Salmonella* Typhimurium is antigenically and genetically similar to *Salmonella* I 4,[5],12:i:-, it remains unknown whether the monophasic expression or lack of the phase 2 flagellar antigen of *Salmonella* I 4,[5],12:i:-

affects their ability to produce biofilms. There are a few published studies investigating biofilm production in *Salmonella* I 4,[5],12:i:- and/or comparing it to *Salmonella* Typhimurium [160, 207, 293]. Barilli et al., showed that *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- isolated from pig carcasses from slaughterhouses in Italy, were all non-biofilm producers [293]. This contrasts with our study where *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- were predominantly weak biofilm producers. However, there were only 11 isolates included in their study (7 *Salmonella* Typhimurium and 4 *Salmonella* I 4,[5],12:i:-). In contrast, Seixas et al., revealed *Salmonella* I 4,[5],12:i:- strains (n = 133) from clinical, environmental, and veterinary samples in Portugal were half weak and half moderate biofilm producers at 24 h [206]. This is in contrast to our current study, where there were no moderate *Salmonella* I 4,[5],12:i:- biofilm producers and the majority of *Salmonella* I 4,[5],12:i:- were found to be weak biofilm producers (89%) and a few were non-biofilm producers (~11%) at 24 h. Additionally, Seixas et al. also showed that *Salmonella* I 4,[5],12:i:- were moderate biofilm producers as time increased to 48 h and 72 h. In our study, biofilms were measured only at 24 h [203]. Tassinari et al., similarly observed only moderate biofilm producing *Salmonella* I 4,[5],12:i:- (24 and 48 h) isolated from pig farms (e.g. feces, feed, water and environmental samples) at different production stages [207]. However, the study by Tassinari et al. found no significant differences in biofilm production of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium at any time point. A greater variation in biofilm production was

seen in *Salmonella* Typhimurium strains (weak, moderate, and strong producers) than the *Salmonella* I 4,[5],12:i:- moderate biofilm producers [207]. The variation in *Salmonella* Typhimurium also contrasts our study where, 95% of *Salmonella* Typhimurium were weak biofilm producers and only one was a moderate biofilm producer.

There are several factors that may affect the differences in results between previously conducted studies and the current study. First, *in vitro* biofilm formation assay methods via a 96-well microtiter dish are known to be variable due to external factors (temperature, pH, humidity, nutrients, etc.), as well as human error (pipetting and disrupting the biofilm) as it is a multistep process. Thus, this method is often performed in mechanical and biological triplicate, as we did in our study. Some studies used different media (MHB, LB, and TSB), temperatures (22°C, 35°C, and 37°C) and concentrations of crystal violet (CV for Gram staining, 0.1% CV, and 0.5% CV). The differences in materials and methods may cause contrasting results.

Importantly, the variation in results may also be due to the source type of where the strains were isolated. *Salmonella* are known to persist in the farm environment and the ability of *Salmonella* I 4,[5],12:i:- and other *Salmonella* serovars to produce biofilms is thought to be an important factor. However, biofilm production may be largely dependent on the serovar, external environment, necessity for survival, and/or establishing infection [43, 44, 195, 196, 198, 293]. It is likely that biofilm formation may differ based on the source such as clinical, veterinary, and environmental samples. For example, ~94%

Salmonella I 4,[5],12:i:- strains from Seixas et al. were isolated from clinical samples while 52% *Salmonella* I 4,[5],12:i:- strains from Tassinari et al. were isolated from environmental samples (~27% isolated from feces, and ~21% from feed and water). The majority of *Salmonella* I 4,[5],12:i:- strains in these two studies were moderate biofilm producers. Contrastingly, our *Salmonella* isolates originate from cheek meat and head trim samples of swine carcasses where swine were asymptomatic carriers of *Salmonella*. Therefore, the source may play a key role in the virulence of *Salmonella* strains. Overall, our study indicates that several *Salmonella* serovars isolated from swine have the potential to produce biofilms which may occur on food products and food contact surfaces commonly found in the food processing industry (e.g. slaughterhouses), and consumers' kitchens, threatening the health of consumers. In particular, *Salmonella* Enteritidis is among the most common human pathogen worldwide in terms of outbreaks and human salmonellosis and is often associated with chickens/eggs. In the United States, *Salmonella* Enteritidis was ranked the as the most frequently reported serovar in 2016, followed by *Salmonella* Newport and *Salmonella* Typhimurium. As seen in our study, *Salmonella* Enteritidis are known to be strong biofilm producers, potentially making it more virulent and having a larger implication on public health.

There is limited information on the association between biofilm-related genes and the ability to produce biofilms for *Salmonella.* Similar to our study, Seixas et al. found no significant association of *adrA, csgD,* and *gcpA* with biofilm-positive phenotype (weak,

moderate, and strong biofilm producers) in *Salmonella* I 4,[5],12:i:- strains [206]. Additionally, Yin et al. found that the detection of *adrA, csg*A, *csgB*, *csgD*, *fimH, glyA, luxS, ompR, pfs, sipB, sipC, sdiA* and *sirA* biofilm-related genes in 77 *Salmonella* strains (encompassing 8 serovars, not including *Salmonella* I 4,[5],12:i:-) did not significantly differ between biofilm-positive phenotypes [204]. However, unlike Yin's et al. study, our study found that *sdiA* had a significant effect on biofilm production. The presence of *sdiA* had a positive effect on biofilm formation for certain *Salmonella* serovars such as Derby, Enteritidis, Montevideo and Typhimurium, while the absence had a negative effect on biofilm formation in *Salmonella* serovars I 4,[5],12:i:-, Agona, Alachua, Senftenberg, and Typhimurium.

The *sdiA* gene encodes for a putative acyl homoserine lactone (AHL) receptor and *luxR* homolog called SdiA. SdiA plays a role in quorum sensing in *E. coli* and *Salmonella* by detecting mixed microbial communities and responds to their signal molecules, specifically AHLs produced by other bacterial species [294-297]. There are limited studies focusing on the role of *sdiA* in biofilm production, with most investigating *E. coli*. There are two studies in particular that present contradictory results on the effect of SdiA on the production of biofilms [295, 298, 299]. Lee et al. showed that a *sdiA E. coli* mutant had increased biofilm production when compared to the wild type [299]. However, Suzuki et al. showed that overexpression of *sdiA* in an *E. coli* strain, led to an increase in biofilm production while an isogenic *sdiA* null mutant showed a decrease in production compared

to the wild type [298]. This is comparable to the findings in our study where a positive impact on biofilm formation was seen in *Salmonella* serovars harboring the *sdiA* gene. Given that *Salmonella enterica* is commonly associated with intestinal infections where the microbial population is complex and diverse, our study shows the presence of *sdiA* may be a key factor in enhancing *Salmonella*'s ability to produce biofilms and potentially aid in colonization.

Biofilm production is a complicated and multistep process that is highly regulated by various factors such as numerous signal molecules, structural composition (e.g. exopolysaccarides, O-antigen capsule, BAPs, fimbriae, flagella and curli) and environmental stimuli and stressors (e.g. nutrient levels, pH, and temperature) [200, 201]. The mechanisms by which *Salmonella* I 4,[5],12:i:- persist in swine and their environment remains unknown. However, our study is the first to compare biofilm production of *Salmonella* I 4,[5],12:i:- with other monophasic *Salmonella* serovars to further assess if biofilm production is affected by the lack of a second flagellar antigen. *Salmonella* I 4,[5],12:i:- isolated from swine carcasses were weak biofilm producers that did not harbor the *sdiA* gene, which may be associated with enhanced biofilm production. The results of our study suggest that biofilm formation is not affected by the monophasic expression of *Salmonella* I 4,[5],12:i:- strains but rather the presence of specific biofilm-related genes. The same can be said for the other monophasic *Salmonella* serovars; Agona, Alachua, Derby, Enteritidis, Montevideo, Senftenberg. Thus, the ability to produce biofilms was

not essential for persistence of *Salmonella* in swine, as all *Salmonella* serovars included in our study were successfully isolated from swine carcasses at the end of slaughter.

However, biofilms are still a concern for food safety and hygiene, as they can attach and persistently grow on food products and food contact surfaces under proper conditions and may subsequently lead to human infections through consumption of contaminated food products [197]. More studies are needed on the function and relationship of *Salmonella* biofilm-related genes with biofilm formation to help develop the tools to prevent biofilm-related *Salmonella* infections. Furthermore, it is important to determine the role of biofilm formation in *Salmonella* persistence in swine.

Flagella are important virulence factors of *Salmonella* as they are involved in motility and biofilm production and can contribute to the outcome of a successful infection. Our study analyzed differences in motility and biofilm production between monophasic I 4,[5],12:i:- and biphasic *Salmonella* Typhimurium to determine whether the presence of two flagellar antigens (FliC and FljB) has an advantage over having one flagellar antigen (FljB). As mentioned, no differences in swarming motility were observed between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium suggesting the monophasic expression does not affect that particular motility. We also found that biofilm production was not affected by the lack of a second flagellar antigen as there were weak biofilm producers among *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains. Therefore, the monophasic expression of *Salmonella* I 4,[5],12:i:- does not appear to affect

virulence traits such as swarming motility and biofilm formation in this collection of isolates. However, differences in swimming motility between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium were observed, where *Salmonella* Typhimurium swimming was greater than *Salmonella* I 4,[5],12:i:- This indicates that the presence of two flagellar antigens and/or biphasic expression may provide an advantage in swimming for *Salmonella* Typhimurium. Previous studies have shown a particular advantage in *Salmonella* expressing FliC-flagella versus those expressing FljB. Bogomolnaya et al. (2014) and Horstmann et al. (2017) found that FliC (phase 1 flagellar antigen) was dominantly expressed in *Salmonella* strains over weak expression of FljB (phase 2 flagellar antigen). Additionally, *Salmonella* expressing FliC-flagella demonstrated an advantage in motility dependent invasion, target-site selection during swimming, and outcompeted FljB-flagella expressed *Salmonella* in gut colonization in murine gastroenteritis infection models [285, 300]. Though *Salmonella* I 4,[5],12:i:- only express the FljB flagellar antigen and were the weakest swimmers, this particular serovar remains successful in swine and human infection, suggesting motility and biofilm production are not involved in its ecological advantage in swine.

5.4. Resemblance and relatedness of Salmonella *I 4,[5],12:i:-* isolates to the *European* Salmonella *I 4,[5],12:i:- clone*

Aforementioned, the *Salmonella* I 4,[5],12:i:- isolates in our study share the same sequence type (ST34) and genotypic ASSuT R-type (ampicillin ($bla_{\text{TEM-1}}$), streptomycin

(*strA-strB*), sulfonamides (*sul2*), and tetracycline (*tet*(B)) as the European *Salmonella* I 4,[5],12:i:- clone. In addition to the sequence type and unique genotypic resistance profile, the European *Salmonella* I 4,[5],12:i:- clone may also be distinguished by the genomic deletions/mutations/insertions in the phase 2 flagellar locus region such as the *hin, iroB, flj*A, and most importantly *flj*B genes [79, 83, 84]. The deletion profile of *Salmonella* I 4,[5],12:i:- were identified by mapping each genome to the *Salmonella* Typhimurium str. LT2 reference genome (NC_003197) and confirming the presence/absence of *flj*A, *flj*B, and *iro*B, genes in BLAST. The mapping revealed a ~15 kb deletion gap in the phase 2 flagellar antigen region in all *Salmonella* I 4,[5],12:i:- which included the deletion of *flj*B, *flj*A, and *hin,* along with putative transposases, integrases, inner membrane proteins, and hypothetical proteins. However, the *iroB* gene remained conserved. The deletions of the *flj*A and *flj*B genes and preserved *iroB* were confirmed through BLAST. Therefore, *Salmonella* I 4,[5],12:i:- isolates in our study closely resembled the European *Salmonella* I 4,[5],12:i:- clone based on the deletion profile (conserved *iro*B), genotypic resistance profile (ASSuT), and sequence type (ST34).

The types of mobile genetic elements present in the deleted region are a bit more diverse in *Salmonella* I 4,[5],12:i:- clones isolated worldwide. For example, the European clones have been found to carry AMR genes (ASSuT) in IS26 elements, plasmids, replication genes, and/or orfs (IncH1), whereas the Spanish clone has been found carry to AMR genes (ACSSuT-GSxT) in class 1 integrons and/or plasmids [21, 81, 83]. Using

hybrid *Salmonella* I 4,[5],12:i:- genome assemblies, we detected and visualized insertions of mobile genetic elements within the phase 2 flagellar antigen region. Two distinct *Salmonella* I 4,[5],12:i:- isolates were evaluated: (1) one with the MDR genotype *aadA1*, *bla*$_{\text{TEM-1}}$, *strA-strB*, *sul*1, *sul*2, *tet*(B) and (2) one with the same MDR genotype and additional AMR genes *aadA2*, *aph(3')-Ia*, *bla*$_{\text{SHV-12}}$, *dfrA19*, *mcr*-9, *qnrB*2, and *tet*(D). Both isolates contained a ~28 kbp antimicrobial resistance gene and heavy metal tolerance gene cassette harboring the ASSuT R-type (*bla*$_{\text{TEM-1}}$, *strA-strB*, *sul*2, *tet*(B)) and *merACDERT* mercury operon surrounded by mobile genetic elements. Similar findings were seen in the study by Garcia et al., where *Salmonella* I 4,[5],12:i:- (isolated from Germany, Italy, and Switzerland) identified as European clones showed a ~28 kpb resistance region (ASSuT R-type) integrated in the *fljA-fljB* operon location [79]. In contrast, Garcia et al. discovered mobile genetic elements such as transposons flanked by IS26, plasmid replication genes, and open-reading frames (orfs) typically located on IncH1 plasmids, while our study found transposons flanked by IS1 and several mobile genetic element proteins. It is interesting to see that *Salmonella* I 4,[5],12:i:- isolated in the United States (current study) and different countries in Europe (Garcia et al.) showed a ~28 kbp sized insertion with comparable mobile genetic elements. This further indicates the resemblance of our swine-derived *Salmonella* I 4,[5],12:i:- isolates to the prevalent European clone.

To further assess the genetic relatedness and evolutionary relationship of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium, we evaluated the swine-derived *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium isolates from our current study, and included publicly available *Salmonella* I 4,[5],12:i:- genomes isolated from human and swine with the least allelic differences (HeirCC; Hierarchical Clustering of Core-genome MLST). Our study is the first of its kind to use HeirCC for selection of closely related genomes between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium and allowed us to further determine the genomic relatedness and clonal complexity of *Salmonella* I 4,[5],12:i:- through phylogenetic analyses.

The phylogenetic analyses performed on our collection of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium genomes showed two distinct clades grouped by the antigenic profile and sequence type: (1) ST19 *Salmonella* Typhimurium (Clade A) and (2) ST34 *Salmonella* I 4,[5],12:i:- (Clade B). We hypothesize that antimicrobial and heavy metal use in swine resulted in the emergence of drug resistant and heavy metal tolerant *Salmonella* I 4,[5],12:i:- from *Salmonella* Typhimurium. In our study, we observe two separate descendant groups rather than *Salmonella* I 4,[5],12:i:- descending from the group of *Salmonella* Typhimurium. Therefore, this suggests that the MDR ST34 *Salmonella* I 4,[5],12:i:- in our study did not evolve from the local ST19 *Salmonella* Typhimurium in our study. However, of the 299 *Salmonella* Typhimurium isolates from the USDA only 37 were selected based on AMR profiles. More studies are needed with a

larger representation of *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- before we can exclude this possibility. Future studies should focus on comparing *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:- of the same sequence type.

There were significant differences between ST34 *Salmonella* I 4,[5],12:i:- and ST19 *Salmonella* Typhimurium isolates in our study. ST19 *Salmonella* Typhimurium was isolated throughout the year, whereas the majority of ST34 *Salmonella* I 4,[5],12:i:- were isolated in September and November. *Salmonella* Typhimurium is known to be more commonly isolated from animals including swine and may contribute to the variation seen in months of isolation. On the other hand, the isolation of *Salmonella* I 4,[5],12:i:- in the months of September and November may be due to hogs arriving from same farm at a specific time of day.

While both *Salmonella* Typhimurium and *Salmonella* I 4[5],12:i:- were isolated from cheek meat and head trim, *Salmonella* Typhimurium were mostly isolated from cheek meat and *Salmonella* I 4,[5],12:i:- from head trim. Both serovars showed strains were closely related by the month of isolation and sample type. Furthermore, closely related isolates further formed smaller clusters based on the culture technique (enrichment versus no enrichment). Also, it is important to note that there were clonal *Salmonella* isolates included in the study that were isolated from the same sample and media. These isolates were included because we wanted to include all *Salmonella* I 4,[5],12:i:- that were isolated form the study. Interestingly, *Salmonella* I 4,[5],12:i:- were mainly isolated using

the enrichment culture method (Enriched), whereas *Salmonella* Typhimurium isolation was 50-50 for enrichment culture method and EB Count (no enrichment). Some studies have suggested that selection methods favor or not favor certain *Salmonella* serovars which may bias the results [301, 302]. Due to the genomic similarity between *Salmonella* Typhimurium and *Salmonella* I 4,[5],12:i:-, we do not believe the culture techniques used biased the selection of *Salmonella* I 4,[5],12:i:-.

A distinction was observed between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium strains when looking at the antimicrobial resistance phenotype and genotype. *Salmonella* Typhimurium further formed two clades: (1) all phenotypically MDR and harbored the $bla_{\text{CARB-2}}$, *floR*, *sul*1, and *tet*(G) resistance genes and (2) half phenotypically MDR and harbored the *strA-B*, *sul*1, and *tet* (*tet*(A) or *tet*(D)) resistance genes and the other half phenotypically pan-susceptible but harbored $bla_{\text{TEM-1}}$. On the other hand, all *Salmonella* I 4,[5],12:i:- were phenotypically MDR (ASSuT R-type) harboring $bla_{\text{TEM-1}}$, *strA-B, sul*2, and *tet*(B), while one did not show resistance to ampicillin. Among the *Salmonella* I 4,[5],12:i:-, two separate clades were formed: (1) additionally harbored the *qnrB*19 gene, were isolated in September from cheek meat and head trim, and (2) some harbored the *qnrB*19 gene, most were isolated in November, and mainly from head trim. Overall, it can be observed that the genetic relatedness within *Salmonella* I 4,[5],12:i:- or *Salmonella* Typhimurium serovars was strongly based on their genotypic resistance profiles. *Salmonella* I 4,[5],12:i:- strains also grouped by month of

isolation and sample type. Once again, it is important to note that the month may be largely biased by particular farms since samples were only collected over a two-day period. Other studies have shown that *Salmonella* serovars cluster by farm and geographic location [303, 304].

Our second phylogenetic analysis included the *Salmonella* I 4,[5],12:i:- from our study and selected publicly available *Salmonella* I 4,[5],12:i:- isolated from swine and/or humans. *Salmonella* I 4,[5],12:i:- were predominantly isolated from humans in the United States indicating our swine-derived *Salmonella* I 4,[5],12:i:- strains are closely related to clinical human *Salmonella* I 4,[5],12:i:- strains. The large majority of the *Salmonella* I 4,[5],12:i:- exhibited the characteristics of the European clone: (1) ST34, (2) harbored the *bla*$_{\text{TEM-1}}$*, strA-B, sul2,* and *tet*(B) genes (ASSuT R-type), and (3) showed deletion of *fljB* and *fljA* genes while conserving the *iroB* gene. In addition, all *Salmonella* I 4,[5],12:i:- showed only five allelic differences among each other. HC5 in *Salmonella* are often associated with recent outbreaks or transmission chains, suggesting our *Salmonella* I 4,[5],12:i:- isolates are closely related to circulating European clones in the United States and have been recently transmitted between swine and humans. While ST34 was the predominant sequence type, ST2379 and ST2956 were also observed and formed individual clades with other closely related ST34 genomes. Both ST2379 and ST2956 were characterized as the European clone based on the deletion profile but displayed differences in resistance. Most ST2379 *Salmonella* I 4,[5],12:i:- were isolated from

humans in the United States, presented the European clone resistance genotype, and harbored additional resistance genes. ST2379 was closely related to European and Spanish clone ST34 *Salmonella* I 4,[5],12:i:- genomes that showed presence of multiple resistance genes. In contrast, all ST2956 *Salmonella* I 4,[5],12:i:- displayed both the European deletion profile and resistance genotype and were equally isolated from human and swine in the United States. ST2956 were closely related to MDR ST34 genomes with the same ASSuT profile and non-MDR ST34 harboring the *tet*(B) gene. ST2379 and ST2956 *Salmonella* I 4,[5],12:i:- both branched off ST34 *Salmonella* I 4,[5],12:i:-. However, ST2379 branches off a larger clade of ST34 suggesting continued emergence of *Salmonella* I 4,[5],12:i:-, while ST2956 had fewer common ancestors suggesting ST2956 may have evolved rather than emerged. Overall, the sequence type and geographic location of *Salmonella* I 4,[5],12:i:- had a major influence on the relatedness of genomes. In contrast, source type (human or swine) did not have a significant influence.

Interestingly, there were a few genomes (~2%) with the Spanish clone deletion that exhibited the European clone sequence type (ST34) and resistance genotype (ASSuT R-type). The source type and country of isolation varied among these genomes and were not found to be closely related to each other. In addition, ~1% of ST34 *Salmonella* I 4,[5],12:i:- genomes displaying the European clone deletion were pan-susceptible like the United States clone. Although pan-susceptible ST34 *Salmonella* I 4,[5],12:i:- genomes may seem like a rare occurrence in our study (possibly due to our method of selection),

several studies identified *Salmonella* I 4,[5],12:i:- with different profiles of deletion and resistance further establishing *Salmonella* I 4,[5],12:i:- have repeatedly emerged throughout time worldwide. As seen in this study, ST34 *Salmonella* I 4,[5],12:i:- and ST19 *Salmonella* I 4,[5],12:i:- have distinct AMR phenotypes and genotypes and form separate clades, while ST34 *Salmonella* I 4,[5],12:i:- and ST34 *Salmonella* Typhimurium share more recent common ancestors and have similar resistance profiles regardless of the deletion of the *fljAB* operon in ST34 *Salmonella* I 4,[5],12:i:- genomes. ST34 *Salmonella* I 4,[5],12:i:- and ST34 *Salmonella* Typhimurium also have in common the presence of the chromosomal *tet*(B) gene as opposed to the plasmid-related *tet*(A) gene in ST19 *Salmonella* Typhimurium, which may explain the preserved *tet*(B) in ST34 *Salmonella* I 4,[5],12:i:-. This suggests *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium of the same sequence types may be more closely related than those with different sequence types. The hypothesis that *Salmonella* I 4,[5],12:i:- may have evolved from *Salmonella* Typhimurium may be true within the sequence types such as ST34 *Salmonella* I 4,[5],12:i:- and ST34 *Salmonella* Typhimurium or ST19 *Salmonella* I 4,[5],12:i:- and ST19 *Salmonella* Typhimurium. However, further studies are needed to determine the evolution of *Salmonella* I 4,[5],12:i:- from *Salmonella* Typhimurium within sequence type rather than between sequence types.

Lastly, phylogenetic analyses was performed on all *Salmonella* I 4,[5],12:i:- genomes and *Salmonella* Typhimurium from our study and publicly available genomes to

determine evolutionary relationships between the two serovars. *Salmonella* Typhimurium were largely ST19 with a few being ST213 and ST34. All were isolated from swine in the United States, and similarly to *Salmonella* I 4,[5],12:i:-, *Salmonella* Typhimurium were mostly MDR. However, instead of the ASSuT R-type seen in *Salmonella* I 4,[5],12:i:-, *Salmonella* Typhimurium showed resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT). The ACSSuT genotype was encoded by the $bla_{\text{CARB-2}}$, *floR, aadA*, *sul1,* and *tet*(A) and/or *tet*(G). As previous studies have shown, this is the most common resistance genotype in *Salmonella* Typhimurium. *Salmonella* Typhimurium were also shown to harbor resistance genes not commonly found in *Salmonella* I 4,[5],12:i:-, such as *aph*(3')-Ia, $bla_{\text{CMY-2}}$, *cmlA1, dfrA12, sul3,* and *tet*(M). Furthermore, *qnrB* genes was rarely found in *Salmonella* Typhimurium. Although, *Salmonella* Typhimurium showed different sequence types and genotypic resistance profiles, *Salmonella* Typhimurium had a maximum of 200 allelic differences to *Salmonella* I 4,[5],12:i:-, which is associated with long-term endemic persistence [236]. Another interesting finding is the close genetic relatedness of ST213 *Salmonella* Typhimurium and all ST *Salmonella* I 4,[5],12:i:- and the nested hierarchy with ST19 *Salmonella* Typhimurium. These results suggest they share a recent common ancestor and originate from the same ancestral lineage.

Based on the Hierarchical Clustering of Core-genome MLST and phylogenetic analyses, the *Salmonella* I 4,[5],12:i:- in our study were primarily shown to be closely

related to several MDR human *Salmonella* I 4,[5],12:i:- in the United States. In the early 2000s, the *Salmonella* I 4,[5],12:i:- found in United States were rarely resistant and often were pan-susceptible (United States clone). However, based on several studies (including ours), the MDR *Salmonella* I 4,[5],12:i:- has become more successful in the spread and clonal expansion in the United States [305]. This further shows the importance of understanding the relationship between different sequence types of *Salmonella* I 4,[5],12:i:- and between *Salmonella* Typhimurium. Future studies should also investigate the association between ST34 *Salmonella* I 4,[5],12:i:- and ST34 *Salmonella* Typhimurium as well as ST19 *Salmonella* I 4,[5],12:i:- and ST19 *Salmonella* Typhimurium to determine if emergence of monophasic *Salmonella* strains occurs within rather than between the sequence type.

Salmonella I 4,[5],12:i:- and *Salmonella* Typhimurium genomes included in the phylogenetic analyses were isolated between 2001 and 2021. Our results show ST19 and ST213 *Salmonella* Typhimurium were isolated every year with the most being isolated before 2011. ST34 *Salmonella* Typhimurium was isolated in 2017 and 2019, while ST34 *Salmonella* I 4,[5],12:i:- was isolated earlier in 2007 and gradually increased throughout the years with the majority between the years 2015 and 2017. Interestingly, Elnekave et al. also observed an emerging clade of clinical *Salmonella* I 4,[5],12:i:- isolates isolated from swine in 2015 (United States Midwest farms). Similar to our study, the VDL *Salmonella* I 4,[5],12:i:- isolates were also identified as the European clone based on the

sequence type and genotypic resistance pattern. Similarly, the majority of ST2379 were isolated in 2015 and 2016 (first observed in 2013) and ST2956 *Salmonella* I 4,[5],12:i:- was evenly distributed between 2015 and 2018. Interestingly, our study shows that ST34, ST2379, and ST2956 were largely isolated between 2015 and 2016 which may support our findings of ST2379 and ST2956 emergence from ST34 *Salmonella* I 4,[5],12:i:- (as seen in the phylogenetic analyses).

These findings are merely based on submitted data on the Enterobase and NCBI platform; therefore, our results are highly dependent on who is submitting data. Additionally, the year a strain was 'isolated' is based on the year included during submission, which some users may mistakenly include the year of the study or year of submission rather than the year of isolation. Metadata details are not verified or regulated and may result in mistaken interpretations. Additionally, our study only includes a selection of isolates and is only representing swine- and human-derived *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium with the least allelic differences (via HeirCC) and not *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium overall.

In our study, phylogenetic analyses revealed *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium were closely related based on their ST, country of isolation, source type, and most importantly, resistance genotype. We find that our swine-derived *Salmonella* I 4,[5],12:i:- do not form a single epidemic clade, but rather are related to other

circulating *Salmonella* I 4,[5],12:i:- strains in the United States or other countries in Europe.

5.5. Future work

In the swine industry, the use of antibiotics (e.g. enrofloxacin, ceftiofur, and tetracycline) and heavy metals (e.g. copper and zinc) cause selection pressures on bacterial communities like *Salmonella*. Future studies should aim in determining whether antibiotic and heavy metal selection pressures effect the transformation and integration of mobile genetic elements consisting of AMR/HMT genes into the fljB region of biphasic *Salmonella* Typhimurium. One potential study could perform *in vitro* conjugation experiments to potentially recreate the transfer and integration of mobile genetic elements into the fljB region of pan-susceptible biphasic *Salmonella* Typhimurium. To expand on our current study, the fitness costs of AMR genes, motility, and biofilm production should also be evaluated on knockout *fljB* MDR resistant *Salmonella* Typhimurium strains, only expressing the phase 1 flagellar antigen. This is to further confirm that the lack of FljB does not affect the fitness, motility, and biofilm production of monophasic *Salmonella* Typhimurium strains. Additionally, future studies should also focus on determining if emergence of monophasic *Salmonella* strains occurs within rather than between the sequence type by evaluating the evolutionary relationship of *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium of the same sequence type.

6. CONCLUSIONS

In this research, phenotypic and genotypic traits of wildtype strains of *Salmonella* I 4,[5], 12:i:- were evaluated to determine mechanisms that may have allowed *Salmonella* I 4,[5],12:i:- to evolve and expand in swine production, pork processing, and among consuming households.

Similar to the *Salmonella* I 4,[5],12:i:- European clone, *Salmonella* I 4,[5],12:i:- in our study were identified as ST34, showed deletion of *fljAB* and conserved *iroB*, displayed the ASSuT R-type encoded by the *bla*$_{\text{TEM-1}}$, *str*A-*str*B*, sul2* and *tet*(B) genes, and showed integration of antimicrobial resistance (ASSuT) and heavy metal tolerance genes (mercury) within the second phase flagellar antigen region. Additional HMT genes encoding tolerance to copper and silver were discovered in all *Salmonella* I 4,[5],12:i:- isolates. Several *Salmonella* I 4,[5],12:i:- isolates harbored additional AMR genes, *qnrB19/qnrB2* and/or *bla*$_{\text{SHV-12}}$, encoding decreased susceptibility to fluoroquinolones (e.g. enrofloxacin and ciprofloxacin) and resistance to third generation cephalosporins (ceftiofur and ceftriaxone), respectively. AMR phenotypic and genotypic profiles (MDR to pan-susceptible) as well as heavy metal tolerance genes (heavy metal tolerant to no tolerance) varied greatly among ST19 *Salmonella* Typhimurium. Important differences were observed by the resistance genotype where *Salmonella* I 4,[5],12:i:- commonly harbored the *bla*$_{\text{TEM-1}}$, *sul2*, and *tet*(B) genes in contrast to *Salmonella* Typhimurium that harbored the *bla*$_{\text{CARB-2}}$, *sul1*, and *tet*(A) genes. We found that between serovars, the

genomes clustered by sequence type and within serovars; genomes with similar genotypic AMR profiles clustered more closely together. This may be important in determining common ancestors between *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium. Our study also demonstrated that *Salmonella* I 4,[5],12:i:- do not have any fitness cost related to *qnrB*, $bla_{\text{SHV-12}}$, and *tet* resistance genes and that fitness was greater or comparable to *Salmonella* Typhimurium strains under antibiotic pressure. We also observed the monophasic expression of *Salmonella* I 4,[5],12:i:- does not affect swarming motility nor biofilm production but affects only swimming motility. Though swimming motility was affected, monophasic expression has been shown not to hinder the pathogenicity and virulence of *Salmonella* I 4,[5],12:i:-. Furthermore, phylogenetic analyses revealed that our swine-derived *Salmonella* I 4,[5],12:i:- are mainly related to other circulating human- and swine-derived *Salmonella* I 4,[5],12:i:- strains in the United States.

The results in our study raise public health concerns as *Salmonella* I 4,[5],12:i:- isolated from asymptomatic market-ready hogs are shown to be resistant to four antimicrobial classes, two of which are important in the treatment of human salmonellosis. The acquisition AMR/HMT genes and loss of the *fljB* gene may play an important role throughout the emergence of *Salmonella* I 4,[5],12:i:- and host specificity to swine. Despite the fact that *Salmonella* I 4,[5],12:i:- and *Salmonella* Typhimurium are closely related genetically and phenotypically, variations exist in host targets, virulence, and disease manifestations. Importantly, our study showed that the lack of the second phase

flagellar antigen does not affect the success of *Salmonella* I 4,[5],12:i:- in swine as there has been continued emergence and spread to humans worldwide. The potential trade-off between the lack of the second phase flagellar antigen and resistance to antimicrobials/heavy metals seems to increase the fitness and provides an ecological advantage to *Salmonella* I 4,[5],12:i:-, especially in swine and their environment. Our study helps explain the increasing importance of multidrug-resistant *Salmonella* I 4,[5],12:i:- in the ecology of foodborne illness in the United States. Sound knowledge about *Salmonella* I 4,[5], 12:i:- and potential virulence or competitive selection advantages is essential in developing mitigation strategies to eliminate this serovar on the farm or during processing to prevent dissemination into the food chain; ultimately, preventing *Salmonella* I 4,[5],12:i:- infections in humans linked to swine and pork products.

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