

The Pennsylvania State University

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**ANTIMICROBIAL RESISTANCE IN NON-TYPHODIAL *SALMONELLA* FROM  
HUMAN AND RETAIL MEATS—UNITED STATES, 2009-2018**

A Dissertation in

Public Health

by

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## Abstract

**Background:** Non-typhoidal *Salmonella* (NTS) is a leading cause of foodborne illnesses in the United States. Antimicrobial-resistant NTS infections are associated with more bloodstream infections, longer hospitalizations, and higher mortality. Contaminated foods of animal origin are an important source of NTS infections in human. The imprudent use of antimicrobials in animal agriculture could lead to the emergence and spread of resistant NTS, which can be transmitted from retail meat products to humans. The FDA has established regulations to guide and monitor the use of clinically important antimicrobials in food animals. In recent years, whole genome sequencing (WGS) has become a standard tool in NTS outbreak investigations due to its accuracy and cost-effectiveness. However, little has been done to quantify the association between antimicrobial use in food animals and observed resistance in retail meats. Also, more information is needed on how to better use and interpret the results of WGS in routine surveillance of resistant NTS.

**Methods:** The first study used a subset of the publicly available NARMS national clinical and retail meat datasets from 2009 to 2018 (16,741 isolates from humans and 4,318 isolates from retail meats), which contain isolate level MIC data. Fluoroquinolone sales from 2013 to 2018 in food-producing animals reported by the FDA were used as a proxy for fluoroquinolone use. The second study used all the *Salmonella* Typhimurium data (577 isolates from humans and 106 isolates from retail meats) in the publicly available NARMS national clinical and retail meat datasets from 2016 to 2018. In study 1, the Pearson's correlation was used to examine the correlation between normalized fluoroquinolone sales and the prevalence of quinolone-resistant *Salmonella* in retail meats. Differences in quinolone resistance between years were assessed using chi square tests or fisher's exact tests. In study 2, Staramr (0.5.1) on GalaxyTrakr platform

was used to identify AMR determinants and predictive resistance. Sensitivity and specificity of WGS method were calculated with phenotypic resistance results as the reference. SNP-based cluster analysis was used to examine the genetic relatedness of a collection of MDR and pan-susceptible *S. Typhimurium* isolates recovered from retail chickens.

**Results:** In study 1, the prevalence of quinolone resistant NTS in retail meats was positively but insignificantly correlated with the normalized fluoroquinolone sales in food animals ( $r=0.67$ ,  $p=0.1449$ ); and were also positively and significantly correlated with the prevalence of quinolone resistant NTS isolates from human ( $r=0.92$ ,  $p=0.0002$ ). The increase of quinolone resistant isolates in retail meats since 2016 were mostly related to *Salmonella* Infantis and *Salmonella* Enteritidis. In study 2, the overall sensitivity of WGS was 96.47% and the overall specificity was 100.00%. The disagreement between phenotypic and genotypic results were mostly related to streptomycin. The MDR isolates differed by an average of 73 SNPs from each other, while the pan-susceptible isolates differed by an average of 473 SNPs ( $p<0.0001$ ). The nearest distance between a pan-susceptible and an MDR isolate was 547 SNPs. MDR isolates and pan-susceptible isolates distinctly clustered on a phylogenetic tree.

**Conclusions:** Fluoroquinolone sales in food animals were positively associated with the prevalence of quinolone resistance of NTS in retail meat products and humans. WGS is reliable in predicting antibiotic resistance, and is able to provide genetic information for better understanding the evolution of MDR isolates. Continuous surveillance of antimicrobial use in agriculture and clinical settings with WGS is necessary.

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### List of Abbreviations

AMC	Amoxicillin clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
AST	Antibiotic susceptibility testing
AXO	Ceftriaxone
AZI	Azithromycin
CDC	United States centers for disease control and prevention
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and laboratory standards institute
COT	Trimethoprim-sulfamethoxazole
FDA	United States food and drug administration
FIS	Sulfisoxazole
FOX	Cefoxitin
GEN	Gentamicin
GFI	Guidance for industry
IDSA	The infectious diseases society of America
iNTS	Invasive nontyphoidal <i>Salmonella</i>
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
NAL	Nalidixic acid
NARMS	The national antimicrobial resistance monitoring system

NTS	Non-typhoidal <i>Salmonella</i>
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
QRDRs	Quinolone resistance determining regions
WGS	Whole genome sequencing
SNP	Single nucleotide polymorphism
STR	Streptomycin
TET	Tetracycline
TIO	Ceftiofur
VFD	Veterinary feed directive

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## Chapter 1A: Introduction

### 1.0 Background and Context

#### 1.1 Nontyphoidal *Salmonella*

Nontyphoidal *Salmonella* (NTS) are gram-negative bacteria belonging to the *Enterobacteriaceae* family. They are able to colonize in a broad range of hosts, and to cause infections in both humans and animals (Acheson & Hohmann, 2001; Ferrari et al., 2019; World Health Organization, 2018). Among over 2,600 different *Salmonella* serovars identified to date, NTS infections are illnesses caused by serotypes other than Typhi, Sendai, Paratyphi A, B and C (Acheson & Hohmann, 2001; Gal-Mor et al., 2014). *S. enterica* subspecies *enterica* serotype Enteritidis, *S. Typhimurium*, *S. Newport* and *S. Heidelberg* have been found to be most commonly associated with human infections. Nontyphoidal salmonellosis typically has the clinical symptom of self-limiting gastroenteritis in humans, and though rarely, it can lead to severe illnesses or mortality (Acheson & Hohmann, 2001; Haselbeck et al., 2017). Humans usually acquire NTS infections through direct or indirect contact with infected animals, their feces, foods of animal origin, or contaminated environment (Centers for Disease Control and Prevention, 2019; World Health Organization, 2019).

#### 1.2 The Transmission of NTS

NTS bacteria are found throughout the world in a variety of environments and hosts. According to the One Health perspective, the health of humans is interconnected with the health of other living species on our shared planet (Mackenzie & Jeggo, 2019). In the case of NTS, the constant interactions between people and animals in the shared environment accelerate the spreading of NTS pathogens, including the antimicrobial resistant ones, across species. The

transmission of NTS may take place throughout the entire food chain, from farming and processing activities to food preparation and consumption (Landers et al., 2012). For example, a study found that when grouped with *S. Typhimurium*-positive pigs, half of the *Salmonella*-negative tracer pigs turned culture positive. The pathogens persisted in the physical environment (soil and shelter huts) for up to seven weeks after removal of animals (Jensen et al., 2006). Another study (Zhu et al., 2017) suggested that broiler chicken products may be an important carrier of MDR *Salmonella* and that cross-contamination may occur among chicken carcasses, workers and the environment during the slaughtering process.

Almost 90% of human NTS infections are foodborne (Scallan et al., 2011). Retail meat products have been found to have antimicrobial resistant NTS nationally, indicating high possibilities of the transmission of antimicrobial resistant NTS to humans during the handling and consumption of retail meats (Cui et al., 2005; Chen et al., 2004; Lestari et al., 2009; Mukherjee et al., 2019; V T Nair et al., 2018; White et al., 2001; Yin et al., 2021). Outbreaks associated with NTS infections attributed to animal-derived foods are about twice as likely to be antimicrobial resistant as outbreaks attributed to foods from plants (Brown et al., 2017; Hoelzer et al., 2011). In recent years, disease outbreaks associated with salmonellosis have been traced back to ground beef, ground turkey, raw chicken products and pork (Centers for Disease Control and Prevention, 2018).

### **1.3 Public Health Burden of NTS**

NTS are one of the most important causes of reportable foodborne diseases in the world (Centers for Disease Control and Prevention, 2019; Crim et al., 2015; World Health Organization, 2019). It is estimated that NTS cause 93 million enteric infections and 155,000

deaths each year worldwide (Majowicz et al., 2010). In the United States, there are approximately 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths that are attributed to NTS annually, resulting in medical costs exceeding 400 million dollars each year (Centers for Disease Control and Prevention, 2019).

Although most NTS infections lead to gastroenteritis with common symptoms including diarrhea, abdominal pain, fever, and vomiting, an estimated 5% of infected individuals will develop bacteremia and other focal systemic infections including septic arthritis, pneumonia, meningitis and osteomyelitis, which can be life-threatening (Acheson & Hohmann, 2001; Gordan, 2011; Haselbeck et al., 2017). The bacteria causing these invasive diseases are referred to as invasive NTS (iNTS). In some cases, people may develop long-term complications caused by NTS infections such as irritable bowel syndrome and reactive arthritis (Gradel et al., 2009; Ternhag et al., 2008). Some groups are at higher risks for severe NTS infections. Infants, children younger than 5 years old, adults older than 65, and individuals with compromised immune system, such as HIV-infected population or those with acute malaria, are more likely to acquire severe and invasive nontyphoidal *Salmonella* (iNTS) infections (Crump et al., 2015; Haselbeck et al., 2017; Parisi et al., 2018). Antimicrobials such as ciprofloxacin, azithromycin, and ceftriaxone are indicated for patients with severe NTS illnesses or at risk of iNTS (Shane et al., 2017).

In the United States, about 90% of foodborne illnesses caused by NTS recover without visiting a medical provider, 7% require medical care but not hospitalization, and 1.8% require hospitalization. 98% of NTS cases recovered and 2% led to death (Batz et al., 2014). NTS infections lead to serious economic and social consequences. The United States Department of Agriculture (USDA) Economic Research Service's report of economic burden of major

foodborne illnesses ranks NTS the 1<sup>st</sup> among the 15 major pathogens mentioned, with an estimated \$3.7 billion (2013 dollars) total economic losses per year. These losses include productivity loss, medical and other expenses created by cases with and without physician visit, cases requiring hospitalization, and cases leading to deaths (Hoffman et al., 2015).

#### **1.4 Antimicrobial Resistant NTS**

NTS infections typically resolve without intervention, however, antimicrobials should be considered for severe and invasive illnesses (Shane et al., 2017). The Clinical Practice Guideline developed by the Infectious Diseases Society of America (IDSA) suggests that fluoroquinolones should be considered as the first-line oral antimicrobials for susceptible *Salmonella* infections in adults, and azithromycin a common choice for children. Alternatively, ceftriaxone can also be used as a first-line treatment antimicrobial (Shane et al., 2017).

A rising public health concern is the growing rate of antimicrobial resistant NTS. Antimicrobial resistance (AMR) is the decreased sensitivity of microbes to antimicrobials that are capable of kill or stop the growth of NTS bacteria (Prestinaci et al., 2015). Between 2015 to 2017, an estimated 16% of NTS infections (212,500 illnesses) were resistant to at least one of those clinically important antimicrobials used to treat NTS infections and 2% (20,800 illnesses) were resistant to three or more antimicrobials in the U.S. (CDC, 2019). Resistance to antimicrobials complicates treatment and management. Studies have found that antimicrobial-resistant NTS infections may lead to worse health outcomes such as bloodstream infections, bringing about longer hospitalizations, increased mortality and higher healthcare costs (Angulo et al., 2004; Broughton et al., 2010; CDC, 2019; Helms et al., 2002; Varma et al., 2005).

Antimicrobial-resistant NTS infections can be hard and expensive to treat due to two main reasons. First, failure of initial antimicrobial treatment may lead to worse health outcomes, including more invasive illness, and thus may increase the chance of finding resistant strains in blood (Barza, 2002); Second, resistant NTS isolates are often characterized by higher virulence which could exacerbate invasiveness and result in more severe clinical symptoms in patients (Varma et al., 2015). These complications contribute to higher healthcare costs and longer loss of productivity for patients. Specifically, the invasion-related virulence gene *invA* were found highly prevalent in resistant isolates recovered from food animals and humans (Amini et al., 2010; Higgins et al., 2020). Moreover, MDR NTS isolates were found to have higher carriage of virulence genes, resulting in reduced efficacy of clinically important antimicrobials to treat infections caused by these pathogens (Higgins et al., 2020).

### **1.5 Emergence and Spread of Antimicrobial Resistant NTS**

Antimicrobial resistance in NTS bacteria can occur through natural and acquired resistance. Natural resistance may be intrinsic or induced with the exposure to antimicrobials (Reygaert, 2018). Acquired resistance in NTS is mostly associated with plasmid-mediated horizontal gene transfer (Reygaert, 2018; von Wintersdorff et al., 2016). Also, the bacteria may acquire permanent mutations to its own chromosomal DNA which is conferring resistance to certain antimicrobials.

Mechanisms leading to AMR in NTS can typically be grouped into three categories: (1) antimicrobial-inactivating enzymes leading to the destruction or major modification of the antimicrobials, (2) active efflux pumps which expel the antimicrobials out of the microorganism, and (3) restriction of access to the drug target through substitutions, amplifications or



modification (Akinyemi & Ajoseh, 2017; Cabrera et al., 2004; Munita & Arias, 2016; Valdez et al., 2009). Resistances are genetically encoded. The three types of resistance can be encoded on genes that have point mutations, or genes that are acquired horizontally (Eaves et al., 2004; Frye & Jackson, 2013; Ju et al., 2018; Michael et al., 2006). Point mutations in the promoter region of a gene may cause its overexpression and produce enzymes that inactivate antimicrobials (de Vooght, 2009). Point mutations in genes encoding antimicrobial targets may lead to a target conferring resistance to certain antimicrobials (Eaves et al., 2004; Frye & Jackson, 2013). For example, mutations to the gyrase genes were often found responsible for fluoroquinolone resistance (Giraud et al., 2006; Hopkins et al., 2005; Redgrave et al., 2014).

Antimicrobial resistance traits can be transferred vertically as in inheritance from generation to generation, or they can be transferred horizontally across different species with mobile genetic elements such as plasmids, transposons and integrons (CDC, 2020a; von Wintersdorff et al., 2016). The resistance genes carried on mobile genetic elements, particularly plasmids in NTS pathogens, can be horizontally transmitted through the process of transformation or conjugation. The horizontally acquired genes can encode resistance with different mechanisms mentioned above (Carattoli, 2003; Ferguson et al., 2002; Frye & Jackson, 2013).

In summary, AMR in NTS bacteria can be naturally developed or acquired. Natural resistance may be intrinsic (for example, reduced permeability of the outer membrane and the natural activity of efflux pumps) or induced (endogenous genes express resistance after exposure to an antimicrobial) (Reygaert, 2018). Acquired resistance can happen through horizontal gene transfer involved with mobile genetic elements, most commonly, with plasmids (Carattoli, 2003; von Wintersdorff et al., 2016). Also, the bacteria may acquire permanent mutations to its own

chromosomal DNA. The injudicious use of antimicrobials could lead to increased antimicrobial resistant NTS in several ways: the selection and co-selection of high-level resistance to one or multiple antimicrobials in successive bacterial generations; the selection of hypermutable strains; the promotion of the movement of mobile genetic elements (Angulo & Mølbak, 2005; Blázquez et al., 2012; Reygaert, 2018). Evidence indicated that single or successive selections for a mutant phenotype considerably increased the proportion of mutators in laboratory populations (Mao et al., 1997). Thus antimicrobials may act not only as selectors of antibiotic resistant strains, but also as indirect promoter of resistance (Eliopoulos & Blázquez, 2003).

### 1.5.1 Resistance to $\beta$ -lactams

The most found mechanism of resistance to  $\beta$ -lactams in NTS is the production of beta-lactamases and extended-spectrum  $\beta$ -lactamases (ESBLs), which are enzymes that can hydrolyze the beta-lactam ring structure, yielding beta-amino acids with no antimicrobial effects (Alcaine et al., 2007; Bush & Bradford, 2016; Frye & Jackson, 2013). ESBLs are an evolving group of  $\beta$ -lactamases that are also capable of hydrolyze penicillins, cephalosporins and aztreonam but are inhibited by clavulanic acid (Rawat & Nair, 2010).

Common gene families encoding for beta-lactamases produced by NTS bacteria include *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>, *bla*<sub>PER</sub>, *bla*<sub>PSE</sub>, and *bla*<sub>CMY</sub> (Alcaine et al., 2007; Zhao et al., 2009). Notably, these genes are typically carried on plasmids rather than chromosomes, indicating that they are transferable through conjugation and/or transformation (Li et al., 2007; Zhao et al., 2009). A NARMS study found that *bla*<sub>CMY</sub> and *bla*<sub>TEM</sub> genes were predominantly responsible for resistance to  $\beta$ -lactams in NTS isolates recovered from retail meats in the U.S.

(Zhao et al., 2009). The *bla<sub>CMY</sub>* gene encodes a cephalomycinase that exhibits extended resistance to many beta-lactams including cephalosporins.

Notably, ESBL-producing NTS infections are of particular concern, because they are often found to be co-resistant to other classes of antimicrobials such as aminoglycosides, tetracycline and quinolones, in addition to  $\beta$ -lactam antimicrobials (Winokur et al., 2001).

### 1.5.2 Resistance to Quinolones

Resistance to quinolones in NTS has been found linked to two main mechanisms. The first mechanism is chromosomally mediated mutations occurring at the quinolone resistance determining regions (QRDRs). QRDRs resistance to quinolones is attributed to the mutation of the genes that code for DNA gyrase (most cases *gyrA* and few cases *gyrB*) and topoisomerase IV (*parC*), which are the targets for quinolones in bacterial cells (Griggs et al., 1996; Jacoby et al., 2014; Velge et al., 2005; Wang et al., 2017). The aforementioned mutations alter the target site structure and reduce the binding efficiency of quinolones (Acheampong et al., 2019; Redgrave, 2014).

The second mechanism involves plasmid-mediated quinolone resistance (PMQR) (Maka & Popowska, 2016; Jacoby et al., 2014). Compared to chromosomally mediated quinolone resistance, PMQR has been reported less and more recently. PMQR contributes to the resistance of NTS strains to quinolones through three different routes: 1) quinolone resistance proteins encoded by *qnr* genes (*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*) that shield DNA gyrase from the effect of quinolones; 2) *aac(6')-Ib-cr* encodes a variant aminoglycoside acetyltransferase with two amino acid alterations, which inactivate ciprofloxacin and norfloxacin through the acetylation of their piperazinyl substituent; and 3) *OqxAb* and *QepA* encode efflux pumps that extrude quinolones

(Jacoby et al., 2014; Strahilevitz et al., 2009). Although these PMQR determinants are reported to confer low-level resistance to quinolones, the presence of PMQR (particular *qnr* genes) may pose a selection pressure of NTS bacteria when exposed to quinolones and facilitate development of high-level chromosomal quinolone resistance (Jiang et al., 2014; Strahilevitz et al., 2009).

### **1.5.3 Resistance to Azithromycin**

Resistance to azithromycin in NTS is rare. According to the NARMS report, around 1% of NTS isolates from humans showed resistance to azithromycin and none of isolates from retail meat were resistant to azithromycin (CDC, 2016). Azithromycin resistance mechanisms may include 1) the presence of macrolides/azalides resistance genes *mphA*, *mphB* or *mefB*, 2) modifying enzymes, 3) mutations in *rpl* and *rpl* genes encoding ribosomal proteins L22, L4 and 23S rRNA, 4) target site modification by methylases encoded by *erm* genes (e.g. *ermA*, *ermB*, and *ermC*), 5) or the acquisition of efflux pumps (Leclercq, 2002; Nair et al., 2016; Ojo et al., 2004; Wang et al., 2017).

## **1.6 Strategies to Combat the Spread of Antimicrobial Resistant NTS**

### **1.6.1 Regulations on Antimicrobial Use in Food animals**

The use of antimicrobials in poultry and other food animals has contributed to the development of drug-resistant NTS among animals (CDC, 2019; Van Boeckel et al., 2017; Witte, 2000). Recognizing the threat of antimicrobial resistant NTS from food animals to public health, the U.S. Food & Drug Administration (FDA) has developed regulations to guide the use of antimicrobials in animal agriculture. Important regulatory changes in recent years include withdrawing fluoroquinolones from use in poultry in 2005, prohibiting certain extralabel uses of

cephalosporins in 2012, eliminating the use of medically important antimicrobials for growth promotion and feed efficiency in food-producing animals, and bringing the use under veterinary oversight and prescription in 2017 (FDA, 2020c).

Specifically, in response to an observed increase of quinolone resistance in human *Campylobacter* isolates associated with poultry consumption, the FDA withdrew the approval of fluoroquinolones for use in poultry in 2005 (Nelson et al., 2007). Currently, there are two fluoroquinolones, danofloxacin and enrofloxacin, approved for use in swine and cattle under a prescription from a veterinarian (FDA, 2021). There are two cephalosporins currently approved for the treatment and control of diseases in food-producing animals: cephapirin and ceftiofur (FDA, 2021).

In 2015, approximately 70% of the total clinically important antimicrobials in the U.S. were sold for use in food-producing animals (FDA, 2020d). Since the implementation of Guidance for Industry (GFI) #213 in 2017 (FDA, 2017), clinically important antimicrobials are no longer allowed to be used for growth promotion or feed efficiency in animal husbandry. All medically important antimicrobial drugs used in animal feed which used to be over-the-counter (OTC) have been changed to Veterinary Feed Directive (VFD) marketing status. Clinically important antimicrobials used in water and feed for therapeutic purposes now need veterinary oversight and prescription. The sales of antimicrobials used for food animals dropped dramatically since that, with antimicrobials for growth promotion reached to zero in 2017 (FDA, 2020d).

The recent Veterinary Feed Directive (VFD) rules authorized by the FDA also highlighted the importance of the appropriate use of antimicrobials in animal agriculture. Public health interventions that are natural, safe and environmentally friendly are needed to reduce the

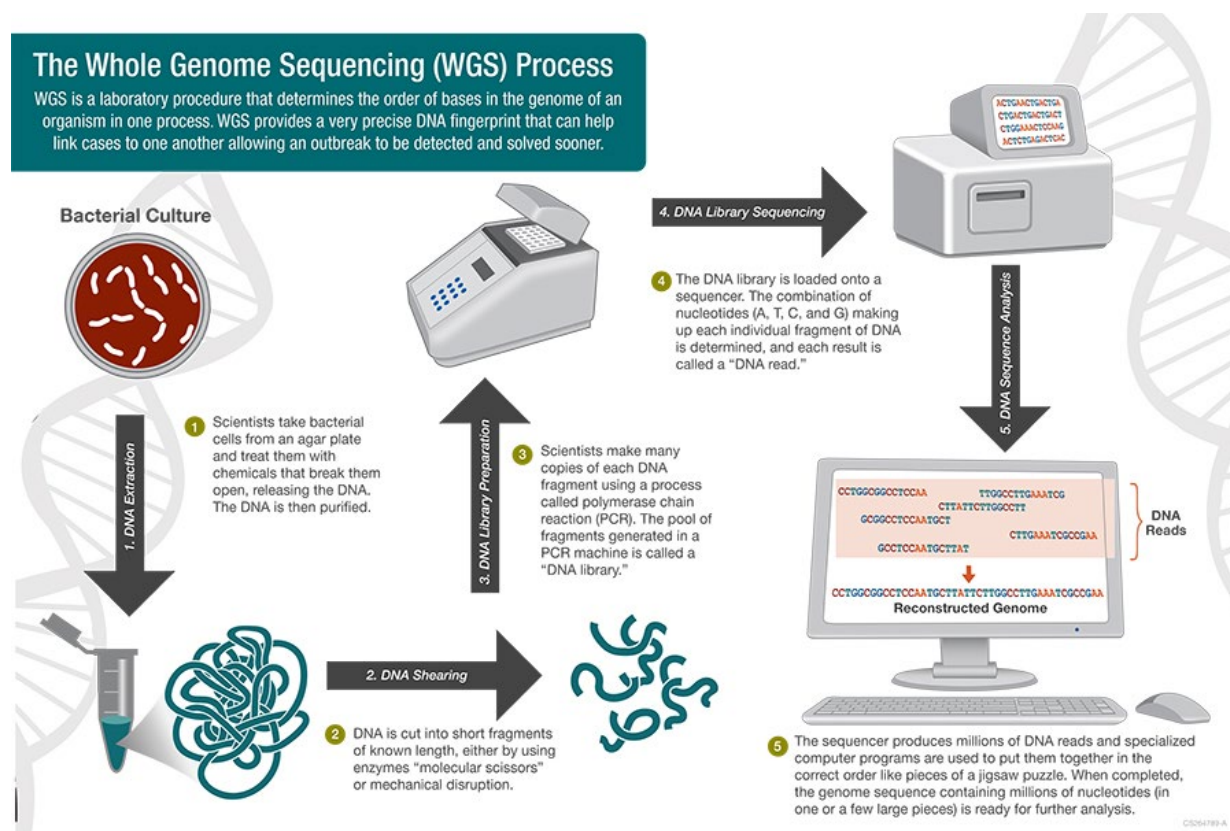
spread of foodborne pathogens such as NTS (FDA, 2020a). However, more research on antimicrobial resistant pathogens in food-producing animals and meat products are needed to assess the impact of these policies. Also, more efforts on detailing the guidance and instruction for antimicrobial use in food animals are needed to ensure the implementation of legislations. Additionally, to help consumers make informed purchase decisions with accurate information on how animals are raised and processed, clear regulations should be established to standardize antimicrobial-related labels or food claims on product packages. Particularly, the USDA Process Verified Program can help ensure the transparency and accountability of production practices.

### **1.6.2 The NARMS Program**

The NARMS program was initiated in 1996 by the FDA's Center for Veterinary Medicine (CVM), the USDA, and the CDC to track antimicrobial susceptibility of foodborne pathogens, including *Salmonella*, found in human (CDC), retail meats (FDA), and food animals (USDA) in the United States (FDA, 2020c). The FDA NARMS program conducts meat sampling in 24 states across the country in collaboration with state public health departments, universities, and other laboratories (FDA, 2019b). Despite this well-established monitoring system, experts have called for the development of more collaborative sites across the country to expand data collection (Ginevan, 2002). Further calls were made to establish electronic systems to enhance data reporting and sharing (Ginevan, 2002; Karp et al., 2017). Finally, environmental testing data were suggested to be added to the program to help further understand the emergence, persistence, and spread of AMR across species (Karp et al., 2017; FDA, 2020a).

### **1.6.3 Whole Genome Sequencing for Detecting and Monitoring AMR in NTS**

Whole genome sequencing (WGS) has emerged as an advanced public health investigating tool for foodborne pathogen monitoring, foodborne outbreak investigations, and AMR surveillance. WGS is a laboratory procedure that determines and analyzes the entire genomic DNA sequence of a cell from an organism, providing comprehensive characterization of the genome (CDC, 2013). The workflow of WGS generally follows five main steps: first, scientists extract DNA from bacterial culture; second, DNA is cut into fragments by molecular scissors or mechanical disruption with known length suitable for the sequencing machine to read; third, DNA fragments are bar-coded and amplified using polymerase chain reaction (PCR), forming a pooled DNA library; fourth, the DNA library is uploaded into a whole genome sequencer and the combination of nucleotides of each DNA fragment is determined; fifth, the sequencer reconstructs DNA reads by putting them in the right order for further analysis (Gautam et al., 2019).

**Figure 1** The WGS Process (CDC, 2013)

Specifically, when applied to characterize NTS isolates in the NARMS program, isolates were sequenced using version 2 or 3 chemistry with paired-end 2- by 250-bp or 2- by 300-bp reads on the Illumina MiSeq platform. PulseNet standard protocols were followed in preparation of DNA libraries, purification, and quality controls and previously described methods for isolates from patients and food sources (CDC, 2017; Tate et al. 2017). De novo assemblies were produced using shovill v.1.0.4. Assemblies were then screened for resistance determinants using staramr v.0.4.0, which employs the reference database from the FDA such as the publicly available resistance gene databases ResFinder [DTU] and CARD [McMaster University]. Resistance genes are determined if they meet the criteria of  $\geq 95\%$  amino acid identity and  $\geq 60\%$  sequence length identity to known resistance proteins.



Before the introduction of WGS in investigating the epidemiology and AMR patterns in foodborne pathogens, NTS isolates were identified and characterized through various traditional methods such as real-time PCR, biochemical assays, phenotypic microarrays (Omnilog), serology and antimicrobial susceptibility testing (AST). Over the past two decades, pulsed-field gel electrophoresis (PFGE) has been the gold standard subtyping method used to discriminate select *Salmonella* serovars and suspected outbreak isolates (Chattaway et al., 2019; Ribot et al., 2019). The traditional approach that involves multiple laboratory techniques to characterize *Salmonella* was very laborious, time consuming and subject to more operational and interpretation error. Moreover, PFGE method can only compare bacterial genomes using 15-30 bands that appear in a PFGE pattern, while WGS allows scientists to compare millions of bases, thus provides much more detailed and precise data.

The advantages of WGS technique are obvious for its streamlined laboratory processes, reduced labor and processing time (without time-consuming culturing as required in phenotypic testing), and improved data accuracy for disease surveillance and outbreak investigations in real time (Chattaway et al., 2019; Punina et al., 2015). The cost of sequencing a 5-megabase pair-sized bacterial genome is about \$120 in today's public health laboratories (Besser et al., 2018). Though the cost is still higher than PFGE (\$30), WGS could provide cost savings with its advantage of offering a universal subtyping and reference characterization system, which consolidates pathogen identification, virulence, and antimicrobial resistance testing, into a single genomic workflow (Kubota et al., 2019). In addition, with the application of WGS, it enables laboratories to identify pathogens directly from primary specimens within hours without culturing the organism (Kubota et al., 2019). Recent studies have supported the effect of WGS approach in helping with NTS outbreak investigations, understanding its

pathogenicity, resistance mechanism, evolution and developing pattern, which contribute to informing public health interventions aimed to reduce NTS infections, as well as the spread of antimicrobial resistant NTS (Chattaway et al., 2019; den Bakker et al., 2014; McDermott et al., 2016; Pornsukarom et al., 2018; Punina et al., 2015; Taylor et al., 2015).

## **2.0 Problem Statement**

Non-typhoidal *Salmonella* (NTS) is a major cause of foodborne illnesses throughout the world (CDC, 2019; World Health Organization, 2019). Antimicrobial-resistant NTS infections are associated with more adverse health outcomes, longer hospitalizations and higher mortality (Angulo et al., 2004; Helms et al., 2002; Martin et al., 2004; Mukherjee et al., 2019; Parisi et al., 2018; Varma et al., 2005). Food animals are important reservoirs of NTS pathogens, and antimicrobial resistant NTS in food animals can be transmitted from retail meat products to humans (Tollefson et al., 1997; V T Nair et al., 2018). Since the injudicious use of antimicrobials in food animals could lead to the emergence and spread of resistant NTS, clinically important antimicrobials including fluoroquinolone in food animal agriculture should be used judiciously. To address this, the FDA has established regulations to guide and monitor the use of clinically important antimicrobials among food animals. However, the associations of fluoroquinolone use in food animals with the prevalence of quinolone resistant NTS in retail meats and humans remain unclear. The profile of AMR in important NTS serovars, such as *S. Typhimurium*, recovered from retail meats and humans is necessary to further understand antibiotic resistance, resistance mechanism, diffusion and evolution. Also, more evidence is needed to assess the reliability of WGS to provide accurate and detailed genotypic information of NTS isolates.

### **3.0 Purpose Statement**

The specific aims and hypotheses of this research are:

1. In study 1, to examine the association between fluoroquinolone sales in food animals and the prevalence of quinolone resistant NTS isolated from retail meats in the U.S. using NARMS data collected during 2009-2018; to examine the association between fluoroquinolone resistant NTS isolated from retail meats and the prevalence of fluoroquinolone resistant NTS isolated from humans using the same data. We hypothesize that there would be a positive correlation between fluoroquinolone sales for use in food animals and the prevalence of quinolone resistant NTS in retail meats; and that the prevalence of quinolone resistant NTS in retail meats would be positively correlated with that in humans.

2. In study 2, to examine the AMR profile of *S. Typhimurium* recovered from retail meats and humans, using NARMS data during 2016-2018; to examine the correlation between phenotypic and genotypic testing of AMR in *S. Typhimurium* by retail meat types; and to examine the genetic relatedness of a collection of MDR and pan-susceptible *S. Typhimurium* isolates recovered from poultry. We hypothesize that there would be a high concordance between phenotypic and genotypic testing. We also hypothesize that MDR *S. Typhimurium* isolates will have closer genetic relatedness than pan-susceptible ones.

### **4.0 Significance of the Proposed Studies**

The proposed studies will help researchers and regulators understand the associations between drug use in food animals and the prevalence of antimicrobial resistant NTS in retail meats and humans. It will also test the accuracy and effectiveness of WGS to characterize resistant NTS isolates, and provide genetic information to further understand the resistance

mechanism and evolution during routine surveillance and outbreak investigation. The results could emphasize the need for integrated surveillance to track trends in AMR and to detect the emergence of clinically consequential pathogens in humans and food animals.

## Chapter 1B: Leadership

Antimicrobial resistant NTS result in serious economic, health and social consequences in the U.S., making it an urgent public health concern. Public health leaders at national, state, local, and hospital levels are obliged to design and implement effective interventions to curb the emergence and spread of drug resistant foodborne pathogens including NTS. Specifically, the misuse of antimicrobials in food animals contributes to the development of resistant NTS bacteria, which can be transmitted to humans through the food chain. Thus, it is imperative for public health leaders to supervise appropriate use of antimicrobials in the animal agricultural industry and strengthen the monitoring of AMR in NTS from food animals, retail meats, humans, as well as the environment.

### 1.0 Personal Leadership Philosophy

As described in the book *Leadership Theory and Practice* (Northouse, 2016), leadership is a process whereby the leader influences others to reach a common goal. I hold a similar belief that leadership is a process to improve oneself and create environments that bring out the best in people to strive for both individual and team accomplishments. Though my leadership is a continual process that I have been working to develop by learning from others and myself, there are some foundational values and beliefs I hold in terms of what defines a leader, which form my own leadership philosophy. It is my belief that these principals will guide me to live a productive life and at the same time, to positively impact people around me.

The foundational values of my leadership philosophy are integrity, duty, diverse perspectives, competency and collaboration. To earn my team's trust and respect, I require myself to be honest, accountable, open-minded and communicative. First of all, integrity is one

of the core qualities of a leader. A man of righteousness and honesty is the one most likely to earn trust from team members, and trust is the cornerstone of any group work and one of the prerequisites of success. I do not consider integrity merely as a judgement call or a policy. It is being honest, sincere and truthful as always. I believe this is one of the fundamental rules of working in a team.

Second, I place great value in duty—being responsible not only for my own part but for the shared mission we are all working on. When I have a project, big or small, I strive to be a role model, to do anything I can to contribute to the group work and let the team members know that each one of them is making success happen. Even times when I am not the team leader, I am fully aware that my duty is not any less, because any mistakes I make, any problems I ignore or overlook could undermine others' efforts and ultimately, the whole group's work.

Third, a good leader must be open-minded to different perspectives. Coming with an international student background, I especially appreciate that my personal experience from a different culture has been welcomed and valued. I hope that my peers have learned from my perspectives which provided a fresh angle to look at issues. My experience told me that diverse point of views can be of great benefits in problem-solving and can spark fresh ideas when facing stagnation. As a leader, I welcome diverse perspectives and opinions, which I believe is a necessary and beneficial element for a team. Reasonable and respectful debate during discussions should be encouraged, because that will generate alternatives and inspire new ideas to solve a problem or challenge. Each member should try their best to contribute to group meetings by sharing information or opinions that would be beneficial for the leader to make sound decisions.

Fourth, to be able to guide others, a qualified leader has to equip oneself with certain skills, including decisiveness, management techniques and expertise in the target area. These competencies help leaders to solve practical problems and implement plans smoothly. From managerial level to specific technical level, it is necessary for leaders to develop well-rounded competencies in order to set clear visions, evaluate risks, make wise decisions, assign work and duty, monitor progress and instruct with specific issues such as statistical analysis in a quantitative study of diabetes. Action speaks louder than words. Therefore, I sharpen my expertise in both work and study areas to get myself better prepared in leading teamwork. The greater competency the leader has, the more possible the team will succeed under his or her guidance.

Last but not least, collaboration is another value that guides me to be a good leader. Teamwork is essentially about working with each other. It is unavoidable to cooperate and communicate with each other to ensure that each member's part is progressing well, and if not, what to do to solve the problems at hand. Smooth collaboration will also help enrich the resources, enhance work efficiency, and accomplish the project goals in a quicker way. Naturally, leaders are expected to have advanced communicative skills, including both talking and listening skills, so that they can express thoughts clearly, state issues effectively, offer support to each other and build emotional connections among the team.

### **1.1 Guiding Theory: Situational Leadership**

Situational leadership emphasizes the flexibility to choose appropriate leading strategies based on the relationship between the leader and the teammates, and the performance needs of the team. For leaders who undertake situational leadership, he or she must have higher level of

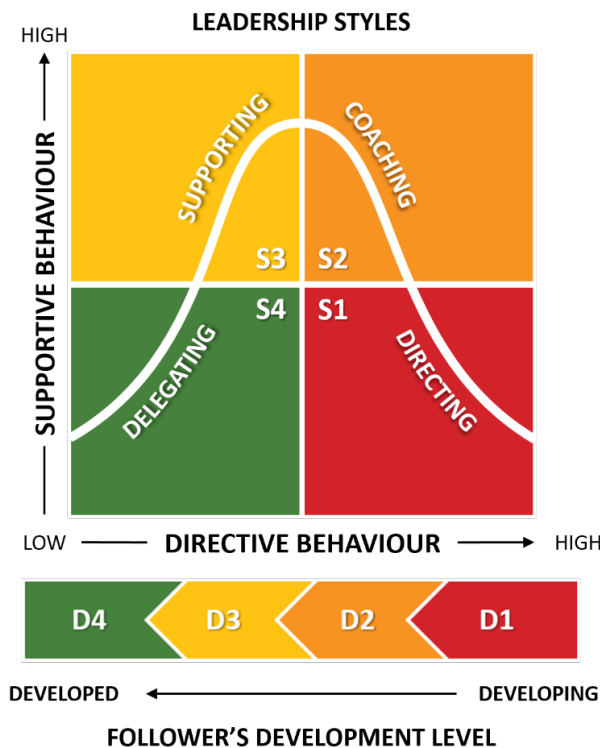
adaptability. It is critical to modify the style of management and communication to fit their goals and circumstances. The different styles of situational leadership can be generally categorized based on two dimensions—directive and supportive behaviors of a leader (Hersey et al., 1979). Directive behaviors include establishing goals, giving directions, defining roles, clarifications, explanations, and assigning jobs. The communication is usually initiated by the leader and teammates are expected to follow directions. Supportive behaviors permit more opportunity for individuals to share thoughts, articulate personal opinions, and build strong relationships among the group (Hersey et al., 1979; Northouse, 2016).

A situational leader adopts one of the following four leadership styles that work best to influence behaviors and enhance performance after carefully considering many variants in teamwork. Style one is a directing leadership style, in which the leader uses higher levels of directive behavior and lower levels of supportive behavior. With this style, the leader provides close supervision and instructions on the tasks for followers who have limited experience, skills or motivations to perform the task. Style two is coaching, which is characterized by the leader using high levels of both directive and supportive behaviors. With this style, the leader oversees the project progress, provides necessary training, and monitor the implementation, but meanwhile, he or she actively recognizes the initiatives, interests and commitment of the teammates for learning, participation and creation. Style three is a supporting style with higher level of supportive behavior and lower level of directive behavior. It is a follower driven style, which can work well if the team has the knowledge and skills to complete the tasks, but just need strong momentum and clear visions. Style four is a delegating leadership style with lower levels of both supportive and directive behaviors. In the case that the team is adequately proficient, motivated and confident to reach the goal, the leader may take this style to create autonomy.



I believe situational leadership theory offers a practical model that highly applicable in real life. Public health interventions to reduce the spread of AMR in NTS will involve cross-sectional and multi-disciplinary collaborations, which requires leaders to be flexible depending on the specific scenario. For example, during the introduction of WGS technique to detect AMR in foodborne pathogens, the directing style may work best to ensure the staff understand the operational steps according to the project objectives. Whereas when developing educational program on food safety, a supporting style may be the most effective, as the team could inspire each other by contributing their ideas about the development of course materials that are informative, motivating and acceptable in local communities. It will also be a valuable chance for team members to strengthen their skills and critical thinking.

**Figure 2** Situational Leadership (Hersey et al., 1979)



## **1.2 Guiding Theory: Transformational Leadership**

Transformational leadership contains four critical elements: idealized influence, inspirational motivation, intellectual stimulation, and individualized consideration (Bass & Riggio, 2006). Idealized influence means that leaders serve as a role model with clear vision and enthusiasm to realize it. By passing on such passion, the leader influences followers to internalize the ideal and formulate positive actions. Intellectual stimulation describes the process of leaders encouraging followers to explore new opportunities, generate deeper understanding and stimulate innovations on certain task. Inspirational motivation relies on a clear vision held and articulated by the leader to his or her followers. The leader is also able to touch the followers and let them experience the same motivation to fulfill their goals. Individualized consideration focuses on personalized support to individual followers and the recognition of the unique contributions brought by them.

Leaders adopting transformational leadership identify needed changes as a meaningful social cause, formulate a bright vision, and strive to realize it together with committed members in a group. Antimicrobial resistant NTS in retail meats may transmit to humans and undermine the treatment of severe NTS infections, bringing considerable social loss and economic burden to individuals and the country, therefore, reducing its spread is a very meaningful and important goal that it could generate public health professionals' inner motivation, enthusiasm and cohesion to make timely responses and implement effective interventions.

**Figure 3** The Four I's of Transformational Leadership (Bass & Riggio, 2006).



## **Chapter 2: Literature Review**

### **1.0 The Prevalence of Antimicrobial Resistant NTS**

In recent years, the emergence of AMR has been found in NTS isolates recovered from human, retail meat products and food animals, raising serious public health concerns. According to the 2016-2017 NARMS Integrated Report (FDA, 2019a), there is an overall increasing trend in NTS resistant to clinically important antimicrobials, particularly ciprofloxacin. In 2017, ceftriaxone resistance in NTS isolated from meat and animals was around 11%, while those from humans was lower at around 3% (Medalla et al., 2017; FDA, 2019a). The percentage of NTS isolates with decreased susceptibility to ciprofloxacin has been rising quickly in both human and meat, reaching nearly 10% in humans and retail chicken meat (CDC, 2019). Although azithromycin resistance in human NTS isolates was rare, the percentage increased from 0.2% to 1.1% in 2017. Azithromycin resistant NTS have not been detected in retail meats and food animals. Microbes which have resistance to three or more antimicrobials are considered to be MDR. MDR NTS have remained around 10% over the last ten years in humans, but increased substantially in isolates from chicken meats and food-producing chicken cecal samples (Medalla et al., 2017; FDA, 2019a). Among NTS serotypes, the ones most commonly found with AMR include *S. Typhimurium*, *S. Enteritidis*, *S. Heidelberg* and *S. Newport* (CDC, 2018; Medalla et al., 2017).

### **2.0 Retail Meat is a Potential Source for Resistant NTS in Humans**

Previous observational studies and foodborne outbreak investigations have supported that retail meats may be a source for antimicrobial resistant NTS in humans. A study conducted in Vietnam (Vo et al., 2006) found the same serotypes and phage types in humans and food animals

with the exception of *S. Enteritidis*, suggesting that food animals may be an important source of human salmonellosis. Zhao and colleagues (Zhao et al., 2003) characterized 87 *S. Newport* strains isolated from humans and food animals in the U.S. Their findings of mobile genetic elements *bla<sub>CMY</sub>* gene (present in all isolates) and class 1 integrons (present in 40% isolates) support the possibility of transmission of *S. Newport* to humans through the food chain. A study in Pennsylvania (M'ikanatha et al., 2010) found multidrug-resistant NTS in poultry meat from retail outlets that carry genes conferring resistance to both ceftiofur and ceftriaxone, indicating that poultry may be a source for human infection of resistant NTS. However, this study had relatively small sample size, and only included poultry products for examination. Another study conducted in Canada using nationwide data from 2003-2008 (Dutil et al., 2010) identified a positive correlation between ceftiofur-resistant *S. Heidelberg* recovered from poultry meats and the incidence of *S. Heidelberg* infections resistant to cephalosporins in humans, implying that the use of ceftiofur in food animals may lead to human infection of ceftiofur-resistant *S. Heidelberg*.

Several foodborne illness outbreaks linked to antimicrobial resistant NTS are reported each year in the U.S. For example, in 2011, MDR *S. Typhimurium*, *S. Heidelberg*, and *S. Hadar* were associated with disease outbreaks. Investigations traced the sources to contaminated ground beef, ground turkeys, and turkey burgers, respectively (CDC, 2018). In 2018, a total of 129 people infected with the outbreak strains of *S. Infantis* from a raw chicken product, resulting in 25 hospitalizations and one death. Isolates from 97 infected individuals had resistance to multiple antimicrobials including ampicillin, ceftriaxone, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin and tetracycline (CDC, 2018). In the same year, outbreaks attributed to resistant NTS pathogens were also traced back to raw turkey products and chicken salad (CDC, 2018).

These lines of evidence suggests the frequent emergence of antimicrobial resistant NTS in the food chain, and highlight the possibility of retail meats as a source for resistant NTS in humans.

### 3.0 *S. Typhimurium* in Retail Meats and Humans

*Salmonella enterica* serotype Typhimurium is the second most common NTS serotype recovered from humans, accounting for 12% of the 16,741 clinical isolates reported to the CDC NARMS, and the most commonly isolated serovar in retail meats, accounting for 17% of the 4,718 isolates reported to the FDA NARMS during 2009 to 2018 (FDA, 2021a). Though on a decreasing trend, *S. Typhimurium* is still among the top three serovars that demonstrate MDR in both humans and retail meats. In particular, MDR definitive phage type 104 (DT104) has been spreading rapidly internationally during the last few decades and causing increased illnesses (Helms et al., 2005; Lan et al., 2009). A more recent WGS study revealed that the dissemination of resistance in *S. Typhimurium* DT104 isolates were related to the genomic island 1 (SGI1) MDR region (Leekitcharoenphon et al., 2016). The authors also found that the susceptible and MDR clusters were genetically distinct, with MDR strains much closer with each other. A recent NARMS study on *S. Typhimurium* isolates recovered from retail meats, humans and food animals (Wang et al., 2019) found that ASSuT (resistant to at least ampicillin, streptomycin, sulfonamides, and tetracycline) and ACSSuT (resistant to at least ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline) were two most prevalent resistance patterns. An earlier NARMS study (Zhao et al., 2005) also detected resistance mostly to streptomycin (63%), tetracycline (61%), ampicillin (61%) and chloramphenicol (36%) among the 588 *S. Typhimurium* from animal origin. These studies together with others (Crump et al., 2011; Glenn et al., 2011; Graziani et al., 2008; Ribot et al., 2002; Zhao et al., 2006) consistently found that *S.*

Typhimurium had very low resistance to fluoroquinolones, a clinically important antimicrobial recommended as the first-line treatment for NTS infections in human (Shane et al., 2017).

#### **4.0 WGS is Effective in Predicting AMR among NTS**

Compared to traditional methods PFGE and AST, WGS has advantages of being cost-effective, time and labor-saving, and being able to render high-resolution genetic information (Chattaway et al., 2019; Ford et al., 2021; World Health Organization, 2018). Previous epidemiological studies (Deng et al., 2015; McDermott et al., 2016; Nair et al., 2016; Neuert et al., 2018; Pornsukarom et al., 2018; Zankari et al., 2013) provided evidence that WGS was highly effective to predict AMR in *Salmonella*. A comprehensive NARMS study (McDermott et al., 2016) reported an overall 99% concordance between phenotypic resistance with known resistance genes of 640 *Salmonella* isolates from retail meats and humans, with disagreement mostly related to streptomycin and cefoxitin. A Pennsylvania study (Keefer et al., 2019) observed 100% correlation among all antimicrobials tested except for streptomycin. Similarly, two U.K. studies identified 97.82% genotype-phenotype correlation (Neuert et al., 2018), and 89.8% concordance (Mensah et al., 2019) respectively, with major errors related to streptomycin and sulfamethoxazole. Notably, using the inaccurate MIC cutoff in phenotypic testing methods could cause the discordance. Specifically, MIC breakpoints to define streptomycin and cefoxitin resistance being too high may cause isolates that carried resistance genes showing phenotypical susceptibility in prior studies (McDermott et al., 2016; Tyson et al., 2016). In addition to the inaccuracy of breakpoints, silent resistance genes, mechanisms attenuating or inactivating gene expression, and the existence of unknown resistance genes may also contribute to the

discordance (Adesiji et al., 2014; Garcia-Migura et al., 2012; Mensah et al., 2019; Vekari et al., 2019).

## 5.0 Summary

Though the link between antimicrobial resistant NTS in human and retail meat is strong, few studies contributed to understand and compare the characterizations, patterns and trends of antimicrobial resistant NTS from human and retail meat products in the U.S. Moreover, to our best knowledge, there has been no study examining the associations between the antimicrobial usage in food animals and the prevalence of resistant NTS in retail meats and humans. Overall, within this topic, most previous studies were concentrated on examining antimicrobial resistant *Salmonella* in meat or human (Cui et al., 2005; Taylor et al., 2015; White et al., 2001; Wang et al., 2017), few compared resistance patterns and resistance genes between isolates from human and meat over the years. Among studies which included isolates from both human and meat products, they are subject to limitations such as small sample size (M'ikanatha et al., 2010; Vo et al., 2006; Zhao et al., 2002), focusing on only one serotype (Dutil et al., 2010; Zhao et al., 2002), or only one type of meat (Dutil et al., 2010; M'ikanatha et al., 2010), and conducting the study outside of the U.S. where there are different patterns of serotypes and phage types for NTS from food animals and human (Dutil et al., 2010; Vo et al., 2006).

Compared to other NTS serovars, *S. Typhimurium* is more prevalent and resistant in retail meats and humans. Its resistance pattern has been evolving and studies with newer, larger dataset is needed to further understand how it persists and distributes across the food chain. Moreover, previous studies around the world have proved WGS an effective tool in *Salmonella* outbreak investigations for detecting AMR and tracing sources (Agren et al., 2016; Chattaway et



al., 2019; Gymoese et al., 2017; Lienau et al., 2011; Octavia et al., 2015; Phillips et al., 2016; Taylor et al., 2015). Few have utilized WGS to examine the genetic relatedness between MDR *S. Typhimurium* isolates and pan-susceptible ones.

Therefore, this research intends to understand the potential association between fluoroquinolones usage in food animals and the prevalence of resistant NTS in retail meats and in humans in the U.S., using 2009-2018 NARMS data. It also aims to depict the AMR profile of *S. Typhimurium* isolates from retail meats and humans using 2016-2018 NARMS data, and examine the genetic relatedness of MDR and pan-susceptible *S. Typhimurium* isolates. Altogether, this research will provide meaningful information for policy makers to reduce the emergence and spread of antimicrobial resistant NTS. It will also provide further context for understanding and interpreting the genetic diversity of *S. Typhimurium* during routine surveillance.

## **Chapter 3: Methodology**

### **1.0 Theoretical Framework**

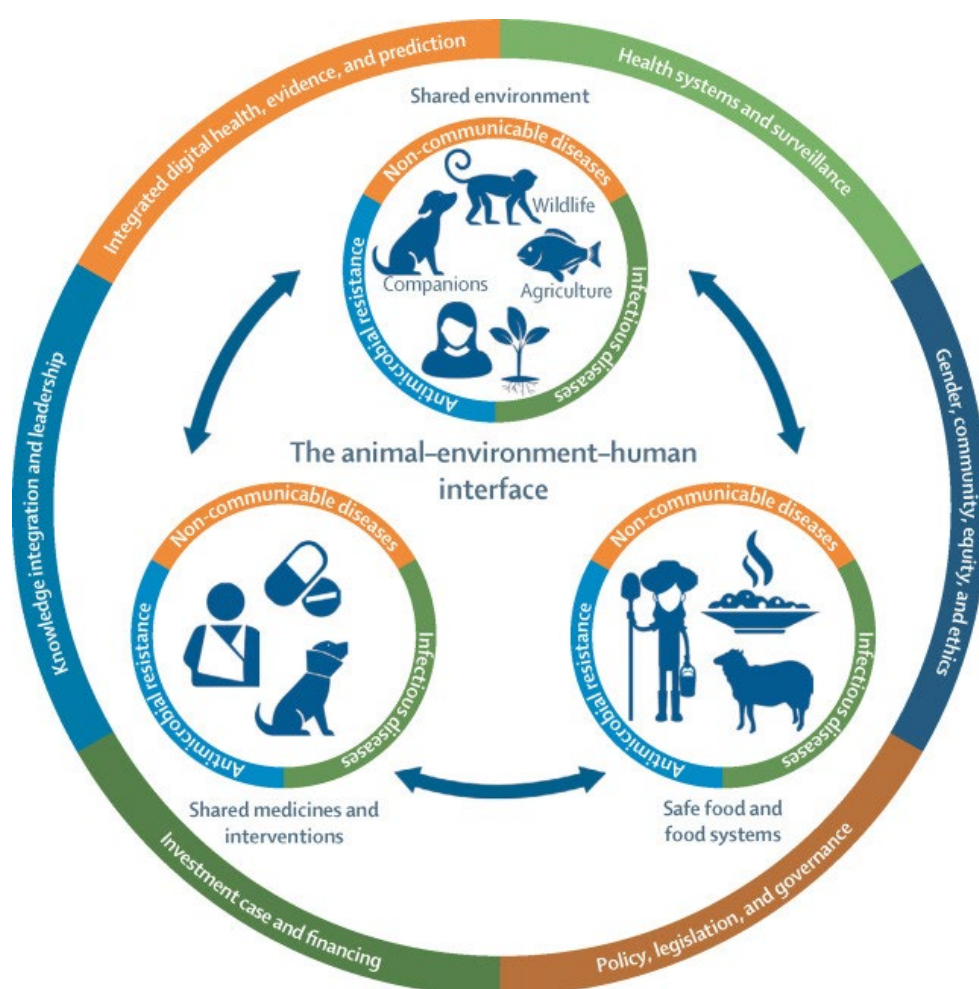
#### **1.1 One Health**

The current research is conceptualized using the One Health perspective. According to the U.S. Centers for Disease Control and Prevention (CDC, 2021), “One Health is a collaborative, multisectoral, and transdisciplinary approach—working at the local, regional, national, and global levels—with the goal of achieving optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment.” The concept of One Health is centered on the recognition that human health is closely connected with the health of animals and our shared environment. The spread of antimicrobial resistant NTS through the food chain reflects the One Health concept (Pokharel et al., 2020). Studies have found the prevalence of antimicrobial resistant NTS in food animal carcasses, and in retail meats, including beef, pork and poultry products, across the country (Cui et al., 2005; Chen et al., 2004; Fakhr et al., 2006; Mukherjee et al., 2019; Wang et al., 2019; White et al., 2001; Yin et al., 2021). PFGE and WGS techniques have revealed comparable genetic characteristics between antimicrobial resistant NTS isolates from retail meats and that from humans (Keefer et al., 2019; M'ikanatha, 2010). These previous lines of evidence indicate that antimicrobial resistant NTS in food animals could transmit to humans through the food chain.

The classes of antimicrobials used for clinical treatment of NTS infections are also used in food-producing animals to prevent, treat and control diseases. A pressing public health concern is that injudicious use of clinically important antimicrobials, such as cephalosporins and fluoroquinolones, among food animals, will facilitate the emergence and

spread of NTS resistant to those drugs. From the One Health perspective, addressing this concern requires multidisciplinary efforts, including integrated surveillance to monitor the development of AMR in humans, retail meats, food animals and the environment, coordinated public health policies at different levels to promote antimicrobial stewardship in human medicine and in food animal agriculture.

**Figure 4** The One Health approach (Amuasi et al., 2020)

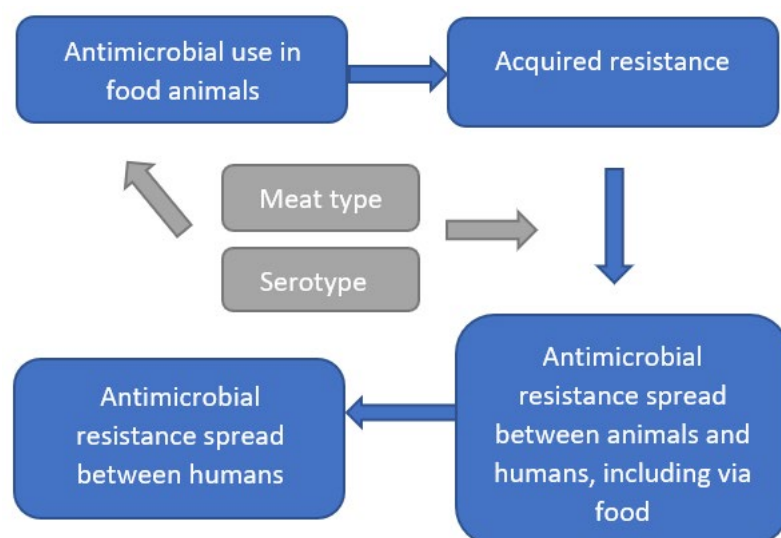


## **1. 2      Health Belief Model**

According to the Health Belief Model, individuals make decisions to change or keep their health-related behaviors based on their perceptions of action barriers and health benefits. For people to take actions, their beliefs in the positive health benefits they may gain must exceed their perceived obstacles or burdens (Champion & Skinner, 2008). The associations between antimicrobial resistant NTS in human and retail meats will likely inform people that retail meat products may be a source for resistant NTS pathogens in human. Based on the Health Belief Model, when purchasing and processing meat products, consumers would consider factors including product prices, their access to these options, trust of information on food packages, convenience of food preparation and taste of the food, then weigh these factors against the health benefits they will get (less possible to acquire antimicrobial-resistant NTS bacteria). Therefore, it is important for policy makers to focus on enhancing availability of various food products options in local communities and promoting food safety education in communities, such as cooking meat thoroughly before consumption, to reduce the likelihood of acquiring AMR NTS infections among consumers.

## 2.0 Conceptual Model

**Figure 5** Conceptual Model



Our assumption is that antimicrobial use in food industry may bring higher prevalence of antimicrobial resistance in food animals, and eventually lead to resistant NTS infections in animals and humans. Meat types and serotypes are considered as confounders because regulation of antimicrobial use is differed by animals and AMR in NTS varied greatly by serotypes.

## 3.0 Study Design

Both studies use the data collected by NARMS which has a design of longitudinal microbiological survey. The benefits of a longitudinal survey include: 1) effective in identifying patterns over time, 2) more power in detecting causal relationships compared to cross sectional studies. The disadvantages of this study design include: 1) require large amount of time to collect data, 2) require a large sample size, 3) relatively more expensive compared to other observational

study designs. However, these main disadvantages are not relevant to the current study since this study is a retrospective study which uses the data collected in the past.

#### **4.0 Sample and Sampling Procedures**

NARMS at the CDC and the FDA monitor the prevalence and trends of AMR among enteric bacteria isolates recovered from humans and retail meats, respectively. Of all the laboratory confirmed NTS infection cases, after serotyping within state laboratories, state health departments will systematically submit every 20th clinical NTS isolates to CDC's NARMS laboratory for antimicrobial susceptibility testing (CDC, 2016). For retail meat NTS sampling, the first step was to purchase retail meat samples from randomly selected grocery stores through collaboration with state health departments and FoodNet sites. Each month, participating sites will purchase about 40 meat samples (including retail chickens, retail ground turkey, retail pork chops and retail ground beef) from grocery stores within randomly selected zip codes. Laboratories will isolate *Salmonella* from retail meats samples according to NARMS Retail Meat Isolation Protocol and send isolates to the FDA for serotyping and antimicrobial susceptibility testing (FDA, 2019b).

### **5.0 Study 1-Fluoroquinolone sales in food animals and quinolone resistance in non-Typhoidal *Salmonella* from retail meats and humans -United States, 2009-2018**

#### **5.1 Study Aims**

This study is aimed at examining the association between fluoroquinolone sales in food animals and the prevalence of quinolone resistant NTS isolated from both retail meat and human clinical isolates. Data from human clinical isolates were collected by CDC NARMS program

while data of NTS isolates from retail meat samples were collected by FDA NARMS program, during 2009 to 2018. Annual fluoroquinolone sales in food animals were obtained from the “Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals” released by the FDA, and were normalized by annual meat production, which were obtained from USDA Economic Research Service (ERS).

## **5.2 Data Source, Data Collection and Management**

This study used a subset of the publicly available NARMS national clinical and retail meat datasets. During 2009 to 2018, 16,741 clinical NTS isolates comprising of 331 serotypes were collected by the NARMS. During the same time, 89,610 retail meat samples, including 35,070 retail chicken parts, 22,456 retail ground turkey products, 16,052 retail ground beef products and 16,032 retail pork chops were collected. A total of 4,318 NTS (2,269 from chicken samples, 1741 from ground turkey samples, 114 from ground beef samples and 194 from pork chop samples) were isolated from retail meat samples, comprising of 78 serotypes.

NTS isolates were tested using broth microdilution (Sensititre®, Trek Diagnostics, part of Thermo Fisher Scientific, Cleveland, OH) to determine the minimum inhibitory concentrations for ciprofloxacin and nalidixic acid (CDC, 2016). Methods for susceptibility testing of NTS and interpretive criteria are described in the NARMS report (CDC, 2016). Using interpretative criteria from the Clinical and Laboratory Standards Institute (CLSI) M100-Ed30 document (CLSI, 2017), NTS isolates were categorized as quinolone resistant if they were resistant to at least one quinolone drug (ciprofloxacin/nalidixic acid). MDR was defined as resistance to at least three or more antimicrobial classes tested by the FDA.

### 5.3 Data Analysis

The Pearson's correlation was used to verify the correlation between quinolone-resistant NTS isolated from retail chicken and human incidence estimates. Differences in quinolone resistance between years using chi square tests. Statistical Analysis Software (Version 9.4) was used for all statistical analyses.

## 6.0 Study 2- Characterization of antimicrobial resistant *Salmonella enterica* serovar Typhimurium with whole-genome sequencing methods

### 6.1 Study Aims

This study is aimed at: 1) characterizing resistant *S. Typhimurium* isolated from retail meats and humans; 2) examining the correlation between phenotypic and genotypic resistance by retail meat types; 3) examining the genetic relatedness of MDR and pan-susceptible *S. Typhimurium* recovered from poultry. The NARMS national clinical and retail meat datasets collected from 2016 to 2018 will be used in this study.

### 6.2 Data Source, Data Collection and Management

This study used NARMS datasets collected from 2016 to 2018 (Available at: <https://wwwn.cdc.gov/narmsnow/> and <https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/narms-now-integrated-data>). We used all *S. Typhimurium* isolates from retail meats and humans. 577 *S. Typhimurium* isolates from humans and 106 *S. Typhimurium* isolates from retail meats were included (SRR accession numbers of all isolates are available at: [https://pennstateoffice365-my.sharepoint.com/:x/g/personal/xzy72\\_psu\\_edu/ET2KQbyija9IpI0pDEMDbIoBzNt\\_DxKI2v9](https://pennstateoffice365-my.sharepoint.com/:x/g/personal/xzy72_psu_edu/ET2KQbyija9IpI0pDEMDbIoBzNt_DxKI2v9)



w5MdvEqi9Cg?e=aEFRyJ). Among those recovered from retail meat samples, 72 were from retail chickens, 8 from retail ground beef, 18 from retail ground turkey and 8 from retail pork chops.

As described in the NARMS report, broth microdilution methods (Sensititre®, Trek Diagnostics, part of Thermo Fisher Scientific, Cleveland, OH) was used to test NTS isolates for susceptibility to 14 antimicrobial agents including gentamicin, streptomycin, penicillin, ampicillin, amoxicillin-clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, azithromycin, chloramphenicol, nalidixic acid, ciprofloxacin, sulfisoxazole, trimethoprim-sulfamethoxazole, and tetracycline (CDC, 2018). CLSI M100 Ed30 criteria and NARMS consensus breakpoints were used to interpret results. NTS isolates that are resistant to three or more antimicrobial classes were considered MDR isolates (Clinical and Laboratory Standards Institute, 2017).

NCBI accession numbers for all characterized isolates were provided in the publicly available NARMS database and were used for WGS analysis. Staramr (0.5.1) on GalaxyTrakr platform was used to scans bacterial genome contigs against the ResFinder, PlasmidFinder and PointFinder databases to identify AMR genes. The criteria used to identify resistance genes were based on a sequence threshold of  $\geq 95\%$  amino acid identity and  $\geq 60\%$  sequence length identity to known resistance proteins.

### **6.3 Data Analysis**

Six hundred and fifty-two isolates with complete antimicrobial susceptibility testing results for all 13 antimicrobials have been included for comparing the phenotypic and genotypic resistance. A total of 8,476 phenotypic data points were generated from the 652 isolates.

Sensitivity and specificity of WGS methods were calculated, with phenotypic resistance results as the reference.

Five pan-susceptible *S. Typhimurium* isolates from poultry were available during the study period. For each pan-susceptible isolate, an MDR isolate were selected from the same year. The collection of 5 pairs of MDR and pan-susceptible *S. Typhimurium* isolates from retail chickens were examined for their genetic relationships. CFSAN SNP Pipeline (Lite) was used to conduct SNP-based cluster analysis and to examine the genetic relatedness of the isolates. FASTTREE (2.1.10) and Newick Display (1.6) were used to construct the phylogenetic tree. Pairwise SNP differences were used to construct heatmap using Complex Heatmap package within R.

## **7.0 Strengths and Limitations**

The strengths of a microbiological survey study design include cost-saving and a large and national representative longitudinal sample. The primary limitation is the sampling bias. For example, sampling of human isolates is more frequent compared to sampling of retail meat isolates. Also, NTS isolates from beef and pork are very limited compared to isolates from poultry. Second, although we used a national dataset, the sample size was still not large enough to examine the prevalence of quinolone resistant NTS isolates by serotype and other confounders. Third, the antibiotics sales and distribution data reported by the FDA do not reflect the actual usage of those drugs on food animals, and do not contain detailed information like drug sales by animal species and label indications. Though sales data are less precise and lack

geographical specificity, we did not find better alternative on antimicrobial use in food animals in the United States.

## Chapter 4: Results and Discussion

### 1.0 Study One

#### **Fluoroquinolone sales in food animals and quinolone resistance in non-Typhoidal *Salmonella* from retail meats and human -United States, 2009-2018**

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**Conflicts of interest/Competing interests:** All authors declare no conflict of interest.

**Institutional Review Board:** Access to clinical *Salmonella* isolates data in the surveillance databases was approved by Centers for Disease Control and Prevention NARMS state-based program activities.

**Patient Consent Statement:** Patient consent to participate is not applicable. Only anonymous isolate level data publicly available on the CDC NARMS website are reported as are genomic data uploaded to NCBI repository.

**Planned Journal:** Zoonoses and Public Health

### Abstract

**Background:** Antimicrobial resistant non-typhoidal *Salmonella* (NTS) infections are associated with worse health outcomes, when compared to susceptible infections. Misuse of antimicrobials in food animals exacerbates the emergence of antimicrobial resistance. Antimicrobials including fluoroquinolone are recommended to treat severe or invasive NTS infections.

**Objective:** The objective of this study was to examine the association between fluoroquinolone sales in food animals and the prevalence of quinolone resistant NTS isolated from retail meats and humans.

**Methods:** We reviewed data from 16,606 human clinical isolates and 4,093 NTS isolates from retail meat samples collected from 2009 to 2018 through the CDC and FDA NARMS programs. Fluoroquinolone sales in food animals were normalized by annual beef and pork meat production. The Pearson's correlation was used to examine the correlation between the prevalence of fluoroquinolone resistant NTS and normalized fluoroquinolone sales; and the correlation between the prevalence of fluoroquinolone resistant NTS isolated from human and retail meats. Quinolone resistant NTS from retail meats were also analyzed by serotypes and meat sources across individual years.

**Results:** Prevalence of quinolone resistant NTS in retail meats was positively correlated with the normalized fluoroquinolone sales in food animals ( $r=0.67$ ,  $p=0.1449$ ); and were also positively correlated with the prevalence of quinolone resistant NTS isolates from human ( $r=0.92$ ,  $p=0.0002$ ). The increase of fluoroquinolone resistant isolates in retail meats since 2016 were mostly related to *Salmonella* Infantis and *Salmonella* Enteritidis.

**Conclusion:** Continued monitoring of fluoroquinolone use in agriculture settings and surveillance for NTS from clinical and food sources is necessary.

## Introduction

Non-typhoidal *Salmonella* (NTS) is one of the most important causes of foodborne diseases in the United States (CDC, 2019). Each year, it is estimated that 1.35 million illnesses, 26,500 hospitalizations, and 420 deaths are attributed to NTS, leading to more than 400 million dollars of medical costs (CDC, 2019). Although most NTS infections result in self-limiting gastroenteritis, an estimated 5% of infected individuals will develop invasive disease which can be life-threatening (Acheson & Hohmann, 2001). In these severe cases, antimicrobial therapy may be necessary for treatment (Shane et al., 2017).

However, there are instances of antimicrobial resistance (AMR) of NTS which complicates treatment. AMR NTS infections are associated with more instances of severe disease and poorer outcomes such as excess bloodstream infections, longer hospitalizations, increased mortality and higher healthcare costs (Angulo et al., 2004; CDC, 2019; Helms et al., 2002; Mukherjee et al., 2019; Varma et al., 2005). Ciprofloxacin, a second-generation fluoroquinolone, is the first-line antimicrobial recommended to treat adult patients with NTS infections (Shane et al, 2017). This class of drugs (fluoroquinolones) are medically important antimicrobials that are not only used in clinical treatment of NTS infections, but also used to treat and control respiratory diseases in food-producing cattle and swine (FDA, 2021). To better monitor fluoroquinolone resistance, the CDC and the FDA also test resistance to nalidixic acid, an early generation of quinolone that is no longer used in clinical treatment, since it may predict the resistance to all quinolones (Albayrak et al., 2004; Sanders, 2001). Despite federal recommendations on cautious usage in human and veterinary medicine, the prevalence of quinolone resistant or non-susceptible NTS in human and retail meats were found to be on the rise in recent years (CDC, 2019; Cuypers et al., 2018; FDA, 2021a).

The increase in AMR NTS may be related to the transmission of AMR NTS from retail meat products to humans (Tollefson et al., 1997; V T Nair et al., 2018; White et al., 2001). Since antimicrobials have been traditionally used in food animals for production and therapeutic purposes, retail meats from animal origin can be important reservoirs of AMR NTS pathogens. Newer recommendations in the implementation of GFI #213 (January 2017) restrict the use of all medically important antimicrobial drugs to treat, prevent and control diseases (FDA, 2017). The overuse and misuse of antimicrobials in agriculture would select resistant strains in food animals (Tollefson et al., 1997; Witte, 2000). For example, a Canadian study has found that ceftiofur use in chicken may be positively associated with ceftiofur resistance in *E. coli* and *Salmonella* isolates recovered from chickens and humans (Dutil et al., 2010). Studies also raised concern on the continued circulation of quinolone resistant *Salmonella* and *Campylobacter* from poultry after the withdrawal of fluoroquinolones (Gupta et al., 2004; Nelson et al., 2007; Price et al., 2007).

Danofloxacin and enrofloxacin are two fluoroquinolones currently approved for use in cattle and swine in the U.S. (FDA, 2021b). According to the Annual Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals released by the FDA, the fluoroquinolone sales has been increasing rapidly since 2013 when data became publicly available (FDA, 2020b). Meanwhile, national surveillance data have revealed an increase in quinolone resistant NTS recovered from retail meats of animal origin over the last decade (FDA, 2021a; M'ikanatha, 2021; Tyson et al., 2017).

The increase in antimicrobial use in food animals, specifically fluoroquinolones which is also used for treatment in humans (i.e., ciprofloxacin), is a major public health concern as it may be contributing to AMR NTS infections (Chantziaras et al., 2014; Endtz et al., 1991; Hopkins et

al., 2005; Piddock, 2002; Sanders, 2001). This study aims to examine the associations of fluoroquinolone use in food animals with quinolone resistance in NTS from retail meats and humans. To examine this relationship, we used a ten-year national dataset containing information of NTS from retail meats and humans collected by the National Antimicrobial Resistance Monitoring System (NARMS).

## Methods

### *Sample Collection, Salmonella isolation, and serotyping*

The National Antimicrobial Resistance Monitoring System (NARMS) aims at monitoring the prevalence of and trends in AMR among enteric bacteria isolates from humans and retail meats at the CDC and the FDA respectively. State and local public health laboratories systematically submit every 20<sup>th</sup> clinical NTS isolate to CDC's NARMS laboratory for susceptibility testing. Retail meat samples were purchased from randomly selected retail outlets by participating NARMS laboratories.

We used a subset of the publicly available NARMS national clinical and retail meat datasets (Available at <https://wwwn.cdc.gov/narmsnow/> and <https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/narms-now-integrated-data>). During the period from 2009 to 2018, 16,741 clinical *Salmonella* isolates were included, comprising of 331 serotypes. During the same time, 89,610 retail meat samples, including 35,070 retail chicken products, 22,456 retail ground turkey products, 16,052 retail ground beef products and 16,032 retail pork chops were collected. A total of 4,318 *Salmonella* (2,269 from chicken samples, 1741 from ground turkey samples, 114 from ground beef samples and 194 from pork chop samples) were isolated from retail meat samples, comprising of 78 serotypes.



### ***Antimicrobial susceptibility testing***

Antimicrobial minimum inhibitory concentration (MIC) data were available for 14 antimicrobial agents within 9 antimicrobial classes including quinolones (nalidixic acid, ciprofloxacin) (FDA, 2019b). Resistance breakpoints were interpreted according to Clinical and Laboratory Standards Institute (CLSI) M100-Ed30 guideline and consensus surveillance breakpoints, where available (CDC, 2016; FDA, 2019b). NTS isolates were categorized as quinolone resistant if they were resistant to at least one quinolone drug (nalidixic acid, ciprofloxacin).

### ***Fluoroquinolone sales***

Fluoroquinolone sales from 2013 to 2018 in food-producing animals were obtained from FDA's 2018 Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals (Available at <https://www.fda.gov/media/133411/download>). Annual meat production data were obtained from U.S. Department of Agriculture Economic Research Service website (Available at <https://www.ers.usda.gov/data-products/livestock-meat-domestic-data/>). The annual total weight of beef and pork production were used to adjust annual fluoroquinolones sales to reflect the estimates of fluoroquinolones use in every kilogram of retail meat.

### ***Statistical analyses***

The Pearson's correlation was used to examine the correlation between normalized fluoroquinolone sales and the prevalence of quinolone-resistant *Salmonella* in retail meats. Differences in quinolone resistance between years were assessed using chi square tests or

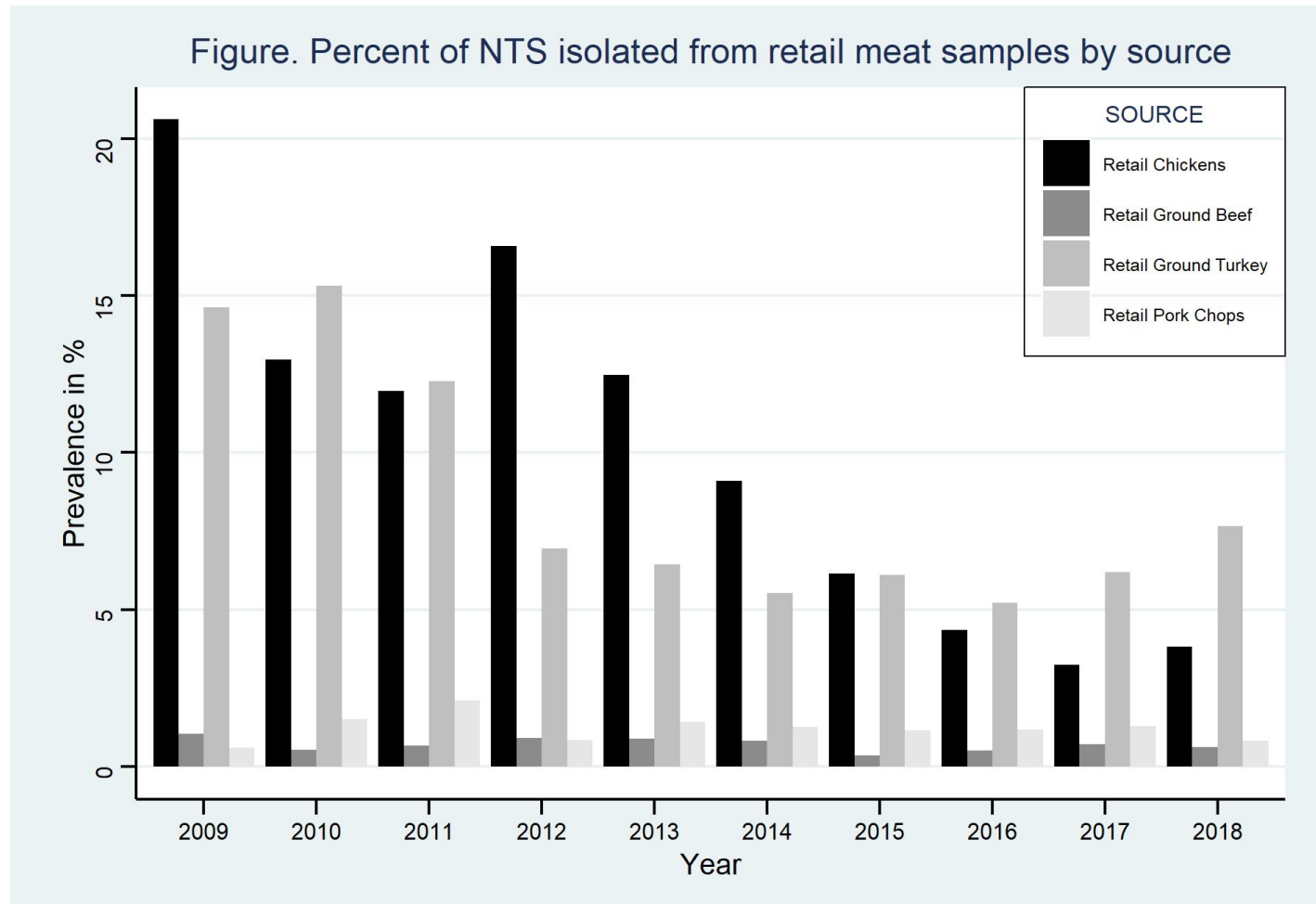
fisher's exact tests. Statistical Analysis Software (Version 9.4) was used for all statistical analyses and a p-value of less than 0.05 was considered statistically significant.

## **Results**

### **Prevalence of *Salmonella* in retail meats**

During the study period, *Salmonella* was detected in 4.82% of total retail meat samples, and was detected in 6.47%, 7.75%, 0.71% and 1.21% of chicken, ground turkey, ground beef, and pork chop samples, respectively. The prevalence of *Salmonella* in retail meats decreased from 11.28% (487/4783) in 2009 to 6.07% (262/5989) in 2014 and then increased to 16.35% in 2018. While the prevalence of *Salmonella* was generally the highest in retail chicken samples from 2009 to 2015, that place has been taken by retail turkey samples since 2016 (Figure 6).

**Figure 6** Percent of NTS isolated from retail meat samples by source



**Table 1** Antimicrobial resistance in non-typhoidal *Salmonella* from humans and retail meat sources United States, 2009-2018

Source	Serovar*	# of isolates	No. (%) of isolates resistant to Quinolones		
			Nalidixic acid	Ciprofloxacin	Quinolones
Human	Enteritidis	3242	337 (10.39)	2 (0.06)	337 (10.39)
	Typhimurium	2003	39 (1.95)	5 (0.25)	39 (1.95)
	Newport	1937	6 (0.31)	0	6 (0.31)
	Javiana	1184	6 (0.51)	0	6 (0.51)
	I 4,[5],12:i:-	755	31 (4.11)	5 (0.66)	31 (4.11)
	Infantis	521	57 (10.94)	2 (0.38)	57 (10.94)
	Montevideo	447	5 (1.12)	0	5 (1.12)
	Muenchen	443	1 (0.23)	1 (0.23)	1 (0.23)
	Other	6209	155 (2.50)	43 (0.69)	164 (2.64)
	All serovars	16741	637 (3.81)	58 (0.35)	646 (3.86)
Meat	Typhimurium	743	1 (0.13)	0	1 (0.13)
	Kentucky	656	1 (0.15)	0	1 (0.15)
	Enteritidis	411	13 (3.16)	0	13 (3.16)
	Heidelberg	344	0	0	0
	Reading	306	3 (0.98)	0	3 (0.98)
	Saintpaul	239	3 (1.26)	0	3 (1.26)
	Hadar	217	0	0	0
	Infantis	188	108 (57.45)	0	108 (57.45)
	Other	1214	12 (0.99)	2 (0.16)	12 (0.99)
	All serovars	4318	141 (3.27)	2 (0.05)	141 (3.27)

\*The eight most common serotypes are listed individually, while other serotypes are grouped into “other” category.

### **Quinolone-resistant NTS isolated from retail meats and humans**

Among the 16,741 clinical isolates, 331 serotypes were identified. The eight most common serotypes accounted for 10,532 (62.91%) of the clinical isolates. While only 58 (0.35%) clinical isolates were resistant to ciprofloxacin, 637 (3.81%) clinical isolates were resistant to nalidixic acid. Among the eight most common serotypes in clinical isolates, Enteritidis (10.39%) and Infantis (10.94%) were most resistant to quinolones (Table 1).

The 4,318 isolates from meat sources had 78 distinct serotypes. The eight most common serotypes accounted for 3,104 (71.89%) of the retail meat isolates (Table 1). Only two *Salmonella* Derby retail meat isolates were resistant to ciprofloxacin. One hundred and forty-one (3.27%) retail meat isolates were resistant to nalidixic acid. Among the eight most common serotypes in retail meat isolates, Enteritidis (3.16%) and Infantis (57.45%) were most resistant to quinolones.

The annual percentage of quinolone-resistant NTS from retail meat samples strongly correlated with the annual percentage of quinolone-resistant clinical NTS ( $r = 0.92$ ,  $p=0.0002$ ) (Figure 7).

### **Temporal changes in fluoroquinolones sales and quinolone resistance**

Fluoroquinolone sales in food animals increased from 15,099 kg in 2013 to 23,350 kg in 2018. After adjusting by annual pork and beef production, fluoroquinolones sales per kilogram meat production increased from 0.72 in 2013 to 1.02 in 2018 (Figure 7).

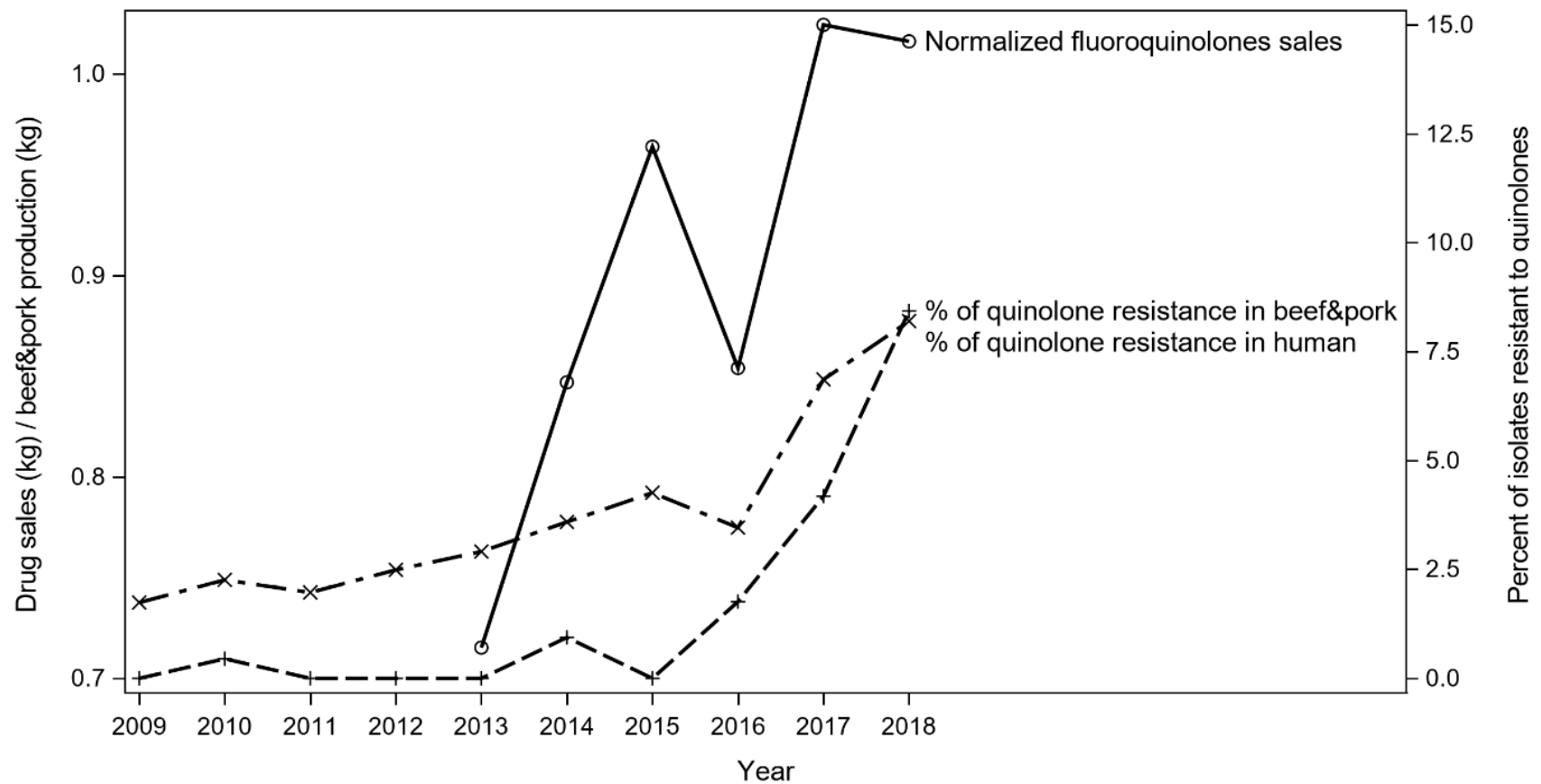
In 2009, 0.62% of *Salmonella* isolates from retail meat samples were resistant to quinolones, and quinolone resistance among clinical isolates was 1.74%. Quinolone resistance remained low from 2009 to 2015 in retail meat isolates and increased sharply from 2016. As a

result, the prevalence of quinolone resistance increased significantly from 0.62% in 2009 to 12.75% in 2018 ( $p<0.0001$ ).

For clinical isolates, quinolone resistance rose slowly from 2009 to 2016 and increased sharply after 2016. The prevalence of quinolone resistant clinical isolates increased significantly from 1.74% in 2009 to 8.20% in 2018 ( $p<0.0001$ )

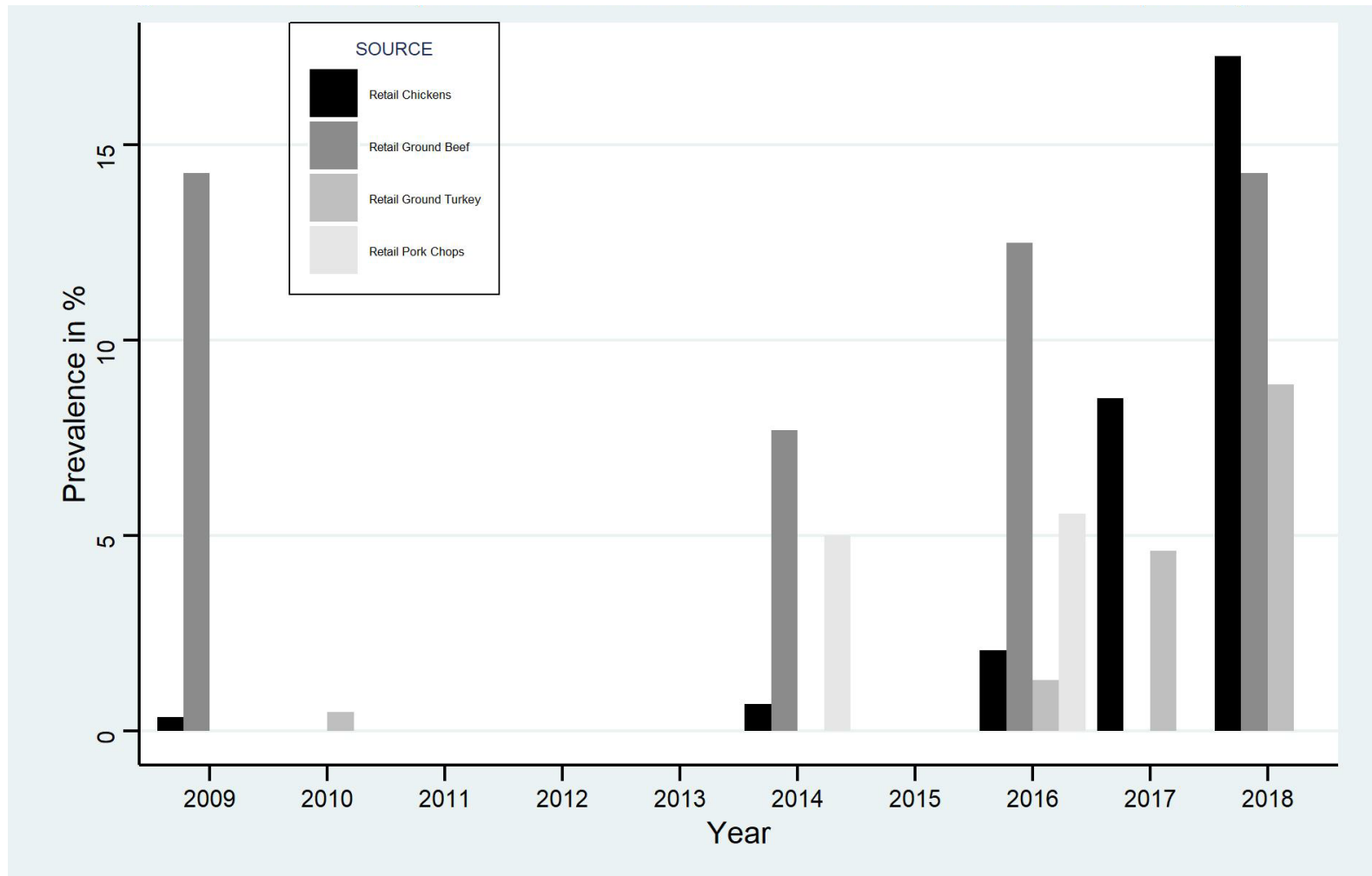
The correlation between the annual (2013-2018) percentage of quinolone resistant NTS from retail meat samples with the annual adjusted fluoroquinolones sales is 0.67 ( $n=6$ ,  $p=0.1449$ ) (Figure 7); or 0.80 ( $n=6$ ,  $p=0.0546$ ) with unadjusted fluoroquinolones sales.

**Figure 7** Fluoroquinolone sales in food animals and the prevalence of quinolone resistant NTS isolates

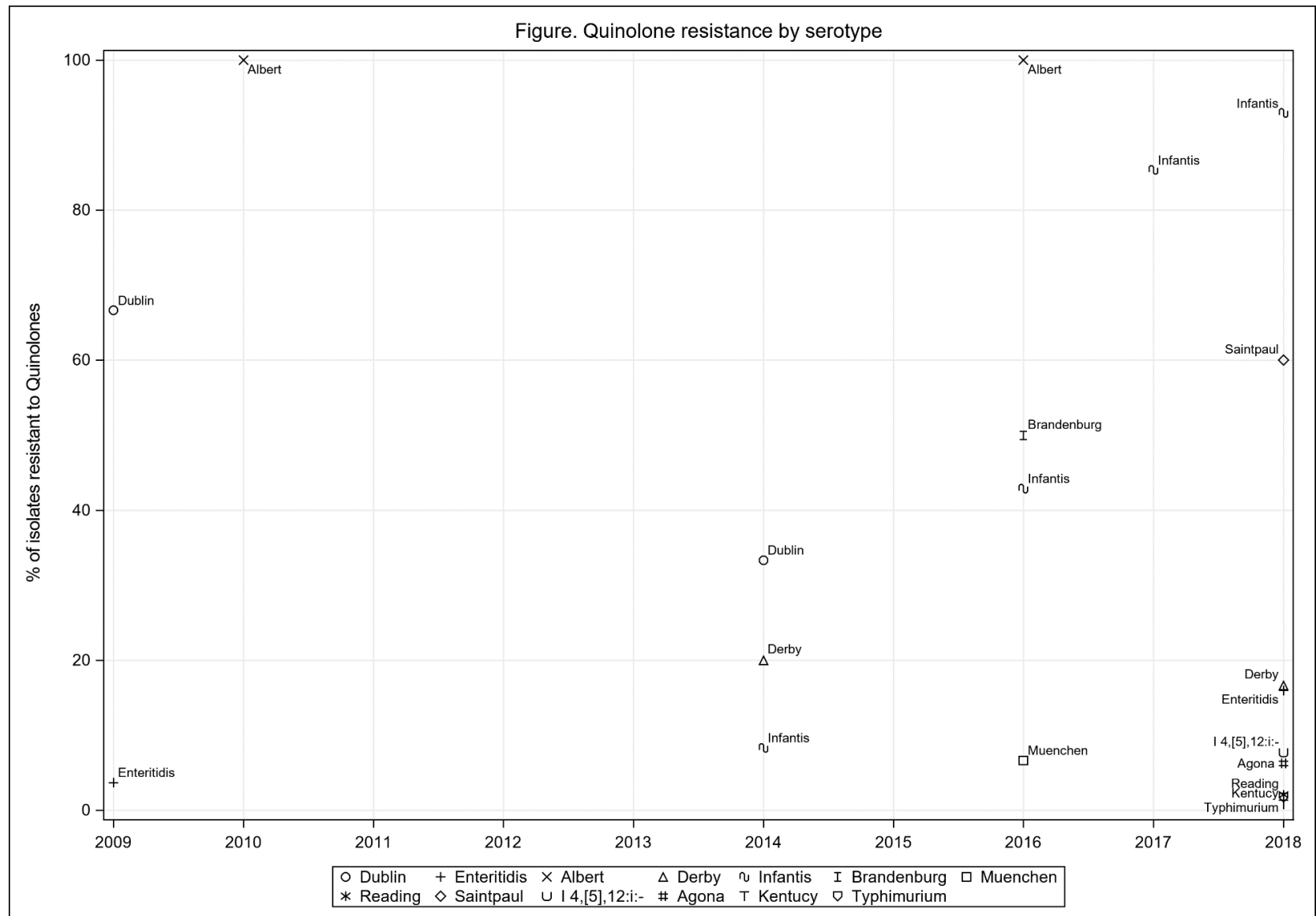


Note: Fluoroquinolone sales before 2013 were not reported by the FDA because there were fewer than three distinct sponsors actively marketing products domestically.

**Figure 8** Percent of quinolone-resistant NTS from retail meat samples by source





**Figure 9** Quinolone resistance by serotype

### Sources and serotypes of quinolone resistant NTS from retail meat samples

Only 0.37% of NTS from retail chicken samples were resistant to quinolones in 2009, compared to 14.29% from retail ground beef samples in 2009 (Figure 8). Also, 0.50% of NTS from retail ground turkey were resistant to quinolones in 2010. There was no other quinolone resistant NTS detected from retail meat samples between 2009 to 2013. From 2014 to 2018, quinolone resistant NTS increased from 0.70% to 17.26% in retail chicken samples ( $p < 0.0001$ ); increased from 7.7% to 14.29% in retail ground beef samples ( $p > 0.99999$ ); increased from 0% to 8.88% in retail ground turkey samples ( $p = 0.0015$ ); and decreased from 5.55% to 0% ( $p > 0.99999$ ) in retail pork chops.

Among the common serotypes in retail meat isolates, the percentage of quinolone resistant *Salmonella* Enteritidis seemingly increased from 3.70% in 2009 to 16.00% in 2018 ( $p = 0.1757$ ); the percentage of quinolone resistant *Salmonella* Infantis increased from 8.33% to 92.96% in 2018 ( $p < 0.0001$ ) (Figure 9).

## Discussion

In this study, fluoroquinolone sales in food animals during 2013-2018 and NTS isolates from retail meats and humans during 2009-2018 were used to analyze the associations of fluoroquinolone use with quinolone resistance in NTS from retail meats and humans. We observed a sharp increase of fluoroquinolone sales in food animals since 2013, along with an increase of quinolone resistant NTS isolates in retail meats and humans. Our results indicate a moderately positive correlation of the annual normalized fluoroquinolone sales with the annual prevalence of quinolone resistant NTS in retail meats; and a strong positive correlation between the annual prevalence of quinolone resistant NTS in retail meats and that in humans.

In the absence of reliable fluoroquinolone use information in food animal production, we used antimicrobial sales data in food animals released by the FDA as a surrogate measure of the level of drug use. The FDA did not release fluoroquinolones sales data in food animals during 2009-2012 because there were fewer than three distinct sponsors actively marketing products domestically (FDA, 2020d). During 2013-2018, the sales of fluoroquinolones in food animals increased 54.65%, from 15,099 kg in 2013 to 23,350 kg in 2018. During the same period, beef and pork production reported by USDA increased 8.85%, from 21,109 kg to 22,979kg (FDA, 2020d). After adjusting for the increase in meat production, normalized fluoroquinolone sales still increased 41.67%. There are currently only two fluoroquinolones (danofloxacin and enrofloxacin) that are approved for use in cattle and swine according to label indications and under veterinarian oversight (FDA, 2021b). In 2012, the FDA amended label indications for danofloxacin and enrofloxacin to control respiratory disease in cattle or swine at high-risk of developing disease in addition to disease treatment (FDA, 2012). Such policy change may partly explain the increase in the normalized fluoroquinolone sales in food animals in recent years.

The prevalence of quinolone resistant NTS from retail meats increased from 0.62% in 2009 to 12.75% in 2018, or 20-fold, and the majority of quinolone resistant NTS from retail meats were resistant to nalidixic acid. Only two isolates, both *S. Derby*, were resistant to ciprofloxacin. However, it is notable that resistance to nalidixic acid, which can be caused by a single mutation within *gyrA*, is a precursor to resistance to all quinolones (Albayrak et al., 2004; Sanders, 2001). Most of the recent increase in quinolone resistance in our retail meat samples were attributed to the *gyrA* mutation (n=366), and especially in *S. Enteritidis* (n=230) and *S. Infantis* (n=51). Only a few (n=57) resistance determinants were found to be plasmid-mediated, including *qnr* genes, *aac(6')-Ib-cr* and *oqxAB*. The number of *S. Enteritidis* with the *gyrA* (D87Y) allelic variant increased from 0 in 2014 to 11 in 2018. For *S. Infantis*, it increased from 1 in 2014 to 66 in 2018. Our finding is consistent with previous studies that quinolone resistance is mostly associated with gene mutations within the quinolone resistance determining regions (QRDRs) (Hooper & Jacoby, 2015; Jacoby et al., 2014; Piddock et al., 2002; Velge et al., 2005; Wang et al., 2017). In specific, we noticed a considerable increase of *gyrA* mutations in *S. Enteritidis* and *S. Infantis* in recent years.

Emergence of *S. Infantis* is a global phenomenon. A recent WGS study (Alba et al., 2020) detected quinolone resistance associated with *gyrA* chromosomal point mutations among 66% of *S. Infantis* isolates collected during 2001-2017 across European countries. The circulation of multidrug resistant *S. Infantis* worldwide in recent years has been reported due to a novel virulence-resistance megaplasmid (pESI, plasmid of emerging *S. Infantis*) (Aviv et al. 2014; Bogomazova et al. 2020; Gal-Mor et al. 2010; Hindermann D, et al. 2017; Tate et al. 2017). However, the resistance to quinolones are chromosomally mediated instead of plasmid

encoded, which highlighted the risk of selective pressure imposed by overuse and misuse of antimicrobials in animal agriculture (Aviv et al. 2014).

The prevalence of quinolone resistant NTS from retail ground beef increased from 5% in 2014 to 11% in 2018. During the same period, we observed a similar steep increase in the prevalence of quinolone resistant NTS recovered from retail poultry, though fluoroquinolones has been prohibited for use in poultry since 2005 (Davis et al., 2009). After reviewing the quinolone resistance in *E. coli* during the same period as sensitivity analysis of the association (Supplemental figure), we saw a similar increase in the prevalence of quinolone resistant NTS from retail ground beef and retail ground pork, but not in retail poultry. This suggests that the increase in the prevalence of quinolone resistant NTS recovered from retail poultry is most likely due to the spread of *S. Infantis*.

During 2013-2018, we observed a correlation of 0.67 between the annual normalized fluoroquinolone sales with the annual prevalence of quinolone resistant NTS in retail meats. This is likely multifactorial and due to the small sample size (n=6), and the lack of fluoroquinolone sale data prior to 2013. Additionally, fluoroquinolone sales are different from the actual usage. They are less precise and lack geographical specificity. Unfortunately, we did not find better alternative on antimicrobial use in food animals in the United States (Scott et al., 2019). Previous studies have demonstrated the utility of using antimicrobial sales data to identify the association between antimicrobial use in food animals and the prevalence of antibiotic-resistant bacteria isolated from those animals (Funk et al., 2006; Harada, 2008; van den Bogaard et al., 2002; Wiuff et al., 2003). Results from a recent randomized control study suggest a significantly higher prevalence of *gyrA* mutations in calves treated with fluoroquinolones compared to the control

group (Pereira et al., 2020), which further corroborated the association we witnessed in our study.

The prevalence of quinolone resistant NTS from humans increased from 1.74% in 2009 to 8.20% in 2018. Although the increase in resistance may be caused by many factors, the use of fluoroquinolones in humans is unlikely to contribute substantially to this, because potent bactericidal drugs like fluoroquinolones are not likely to select for resistance when therapeutic concentrations are obtained (Mølbak et al., 2002). It has been documented that consumption of contaminated retail meat products may lead to the infection of AMR *Salmonella* in humans (Dutil et al., 2010; M'ikanatha et al., 2010; Zhao et al., 2003). This could explain our observation of a strong positive correlation ( $r=0.91$ ) between the quinolone resistance in retail meats and humans. While the mechanism of quinolones resistant NTS are mostly related to gene mutations (*gyr* and *par* genes) within QRDRs, exposure to quinolones in food animals would select and amplify these mutations, thus increase the number of quinolone-resistant NTS strains (Hooper & Jacoby, 2015; Piddock, 2002).

There are several limitations of this study. First, the antibiotics sales and distribution data reported by the FDA do not reflect the actual usage of those drugs on food animals, and do not contain detailed information like drug sales by animal species and label indications. The absence of precise drug use data prevents us from fully understanding the effect of subtle changes in the level and way of using fluoroquinolones in food animals. Second, since the annual sales of fluoroquinolones were first reported in the year of 2013, we have limited data points to adequately illustrate the associations between fluoroquinolones sales and the prevalence of resistant NTS in retail meats. Third, although NARMS data included a relatively large number of NTS isolates recovered from poultry products, we had limited isolates from retail ground beef

and pork, which makes the estimates unstable. Fourth, due to the lack of comprehensive data on the usage of fluoroquinolones in humans, we are unable to fully determine the exact cause of the marked increase in quinolone resistant NTS isolated from humans.

Our findings demonstrated that fluoroquinolone sales in food animals increased simultaneously with the prevalence of quinolone resistance of NTS in retail meat products and humans. Although our data cannot be used to directly attribute the emergence of quinolone resistance NTS in humans to the use of fluoroquinolone in food animal production, the strong correlation suggests that retail meats are potential sources. There is already compelling evidence that widespread use of fluoroquinolones in human and veterinary medicine, combined with non-therapeutic use in agriculture, is fueling the emergence of quinolone resistance (Angulo et al., 2000; Gupta et al., 2004; McEwen et al., 2002; Piddock, 2002; Richard et al., 1994). Previous research also supports that restricting antimicrobial use in food-producing animals is associated with a reduction of resistance in animals, and possibly in humans as well (Cheng et al., 2012; Tang et al., 2017). The results from our study emphasize the need for integrated surveillance to monitor trends in AMR and to detect emergence of clinically consequential pathogens in humans and food animals. Further studies with longer time frame of actual fluoroquinolones use in food animals and humans, and larger sample sizes of NTS from retail meats are needed to understand the precise longitudinal association between fluoroquinolones use and quinolone resistance.

## 2.0 Study Two

### **Characterization of antimicrobial resistant *Salmonella enterica* serovar Typhimurium with whole-genome sequencing methods**

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**Planned Journal:** Food Microbiology



## Abstract

**Introduction:** *Salmonella* Typhimurium is the leading cause of foodborne illnesses in the U.S., causing over a million cases each year. In recent years, whole-genome sequencing (WGS) has become a standard tool for routine epidemiological subtyping.

**Objectives:** The objectives of this study are 1) to compare the phenotypic and genotypic antimicrobial resistance (AMR) profiles of multidrug resistant (MDR) *S. Typhimurium* isolates, 2) to examine the genetic relatedness of a historic collection of MDR and pan-susceptible isolates from retail chickens.

**Methods:** We used data on *Salmonella* Typhimurium isolates in the publicly available NARMS national clinical and retail meat datasets from 2016 to 2018. Staramr (0.5.1) was used to identify AMR determinants and predictive resistance from genomes submitted to NCBI. Sensitivity and specificity of the WGS method were calculated with phenotypic resistance results as the reference. SNP-based cluster analysis was used to examine the genetic relatedness of MDR resistant and pan-susceptible isolates from retail chickens.

**Results:** The overall sensitivity of WGS as a predictor of clinical resistance was 96.47% and the overall specificity was 100.00%. The disagreement between phenotypic and genotypic results were mostly related to streptomycin. The MDR isolates differed by an average of 73 SNPs from each other, while the pan-susceptible isolates differed by an average of 473 SNPs ( $p < 0.0001$ ). The nearest distance between a pan-susceptible and an MDR isolate was 547 SNPs.

**Conclusion:** WGS can reliably predict AMR in *S. Typhimurium* isolates and it can reveal genetic determinants to elucidate the evolution of antimicrobial resistance.

## Introduction

Non-typhoidal *Salmonella* (NTS) is a leading cause of foodborne illnesses in the U.S., accounting for 1.35 million infections, 26,500 hospitalizations, and 420 deaths each year (Centers for Diseases Control and Prevention, 2019). Although most NTS infections are self-limiting, approximately 5% of infections can develop into invasive diseases which may require antimicrobial treatment (Acheson & Hohmann, 2001). Compared to antimicrobial-susceptible infections, antimicrobial-resistant *Salmonella* infections are associated with more bloodstream infections, longer hospitalization, and higher mortality (Angulo et al., 2004; Varma et al., 2005). It is estimated that 212,500 infections from antibiotic resistant NTS occur annually in the U.S., making these infections a serious public health concern (CDC, 2019).

Contaminated foods of animal origin are an important source of *Salmonella* infections in humans (Antunes et al., 2016; CDC, 2020; Wang et al., 2019). Consumption of red meat and poultry has increased steadily in recent decades in the U.S. and per capita consumption has increased 35%, from 75.8 kg in 1960 to 102.1 kg in 2020 (USDA, 2021). Antimicrobials have been used in animal agriculture industry to prevent, control, and treat illnesses in animals (US Food and Drug Administration, 2020). The use of antimicrobials in food animals may select strains resistant to clinically important medicines used in human treatment.

NTS has more than 2600 serotypes, and different serovars may vary with respect to their host specificity and the ability to cause diseases in hosts (Andino & Hanning, 2015; Ferrari et al., 2019). *Salmonella enterica* serovar Typhimurium is the second most common cause of foodborne salmonellosis and the most common serovar isolated from retail meats in the U.S. (Andino & Hanning, 2015; CDC, 2017; FDA, 2016). Moreover, compared to other serovars, *S. Typhimurium* has a high prevalence of multidrug resistance (MDR), especially in retail chickens

and retail ground turkey (Arya et al., 2017; FDA, 2021). Although the prevalence of MDR *S. Typhimurium* recovered from retail meats was decreasing during the recent decade, there were still approximately 40% isolates recovered from poultry samples in 2019 demonstrating MDR (FDA, 2021). The detection of MDR *S. Typhimurium* isolated from retail meats is concerning, as the resistance may be transmitted to humans through the food chain (VT Nair et al., 2018; Wang et al., 2019).

Antibiotic susceptibility testing (AST) is the standard method for detecting and characterizing antimicrobial resistance in NTS, which involves measuring minimum inhibitory concentrations (MIC) of antibiotics. However, AST is subjected to several limitations, such as the ability to test only a restricted number of antimicrobials, inconsistent MIC breakpoints used for susceptibility interpretation (Kahlmeter, 2014; Keefer et al., 2019; McDermott et al., 2016). Also, phenotypic information from AST may not be adequate to determine AMR gene alleles and understand resistance mechanisms. With its higher resolution, simplified sample preparation and lower cost, WGS has now been more widely used in NTS outbreak investigations and source-tracing (Hoffmann et al., 2016; Rounds et al., 2020; Taylor et al., 2015; Vaughn et al., 2020). Prior studies suggested high correlations (91% - 100%) between phenotypic susceptibility testing and genotypic prediction of AMR using WGS (McDermott et al., 2016; Nair et al., 2016; Neuert et al., 2018; Pornsukarom et al., 2018).

Most of the previous studies that compared phenotypic and genotypic resistance were using data of all serotypes and MIC levels that were considered less accurate. SNP based analysis were only used in outbreak investigation or to compare the relatedness between surveillance and outbreak isolates. The National Antimicrobial Resistance Monitoring System (NARMS) in the U.S. tracks antimicrobial resistance in foodborne pathogens including NTS

recovered from humans, retail meats and food animals (Karp et al., 2017). Using a multi-state dataset comprising of three years of clinical and retail meats data collected by the NAMRS, this study intends to: 1) examine the AMR profile of *S. Typhimurium* recovered from retail meats and humans; 2) examine the correlation between phenotypic and genotypic testing of AMR in *S. Typhimurium* with more recent data; 3) examine the genetic relatedness of a collection of MDR and pan-susceptible *S. Typhimurium* recovered from poultry using SNP analysis.

## Methods

### *Salmonella* isolates

We used a subset of the publicly available NARMS national clinical and retail meat datasets from 2016-2018 (Available at <https://wwwn.cdc.gov/narmsnow/> and <https://www.fda.gov/animal-veterinary/national-antimicrobial-resistance-monitoring-system/narms-now-integrated-data>). During that time, 577 clinical *Salmonella* Typhimurium isolates were included ((SRR accession numbers of all isolates are available at: [https://pennstateoffice365-my.sharepoint.com/:x:/g/personal/xzy72\\_psu\\_edu/ET2KQbyija9IpI0pDEMDbIoBzNt\\_DxKI2v9w5MdvEqi9Cg?e=aEFRyJ](https://pennstateoffice365-my.sharepoint.com/:x:/g/personal/xzy72_psu_edu/ET2KQbyija9IpI0pDEMDbIoBzNt_DxKI2v9w5MdvEqi9Cg?e=aEFRyJ)). A total of 106 *S. Typhimurium* isolates were recovered from retail meat samples – 72 were from retail chickens, 8 from retail ground beef, 18 from retail ground turkey and 8 from retail pork chops.

## **Antimicrobial susceptibility testing**

Antimicrobial minimum inhibitory concentration (MIC) data were available for 13 antimicrobial agents within 9 antimicrobial classes (FDA, 2019): aminoglycosides (gentamicin, streptomycin), penicillins (ampicillin),  $\beta$ -lactam/ $\beta$ -lactamase inhibitor combinations (amoxicillin- clavulanic acid), cepheims (cefoxitin, ceftriaxone), macrolides (azithromycin), phenicols (chloramphenicol), quinolones (nalidixic acid, ciprofloxacin), folate pathway inhibitors (sulfisoxazole, trimethoprim-sulfamethoxazole), and tetracyclines (tetracycline). Resistance breakpoints were interpreted according to Clinical and Laboratory Standards Institute (CLSI) M100 Ed30 guideline and consensus surveillance breakpoints (FDA, 2015). MDR was defined as resistance to three or more antimicrobial classes.

## **Whole genome sequencing and resistance genotypes**

NCBI accession numbers for all characterized isolates were provided in the publicly available NARMS database and were used for WGS analysis. Staramr (version 0.5.1) on GalaxyTrakr platform (Gangiredla et al., 2021) was used to scans bacterial genome contigs against the ResFinder, PlasmidFinder and PointFinder databases to identify antimicrobial resistant determinants (Carattoli et al., 2014; Jolley et al., 2018; Zankari et al., 2012, 2017). The criteria used to identify resistance genes were based on a sequence threshold of  $\geq 95\%$  amino acid identity and  $\geq 60\%$  sequence length identity to known resistance proteins.

## **Correlation of susceptibility phenotypes and genotypes**

Six hundred and fifty-two isolates with complete antimicrobial susceptibility testing results for all 13 antimicrobials have been included for comparing the phenotypic and genotypic resistance. A total of 8,476 phenotypic data points were generated from the 652 isolates. Phenotypic resistance results were used as the reference. Sensitivity and specificity were calculated by dividing the number of isolates that were genotypically resistant/susceptible by the total number of isolates exhibiting clinical resistance/susceptible phenotypes.

## **Single nucleotide polymorphism (SNP) analysis**

Five pan-susceptible *S. Typhimurium* isolates from retail chickens were available during the study period. To control for the collection time and source of the isolates, five MDR *S. Typhimurium* isolates from retail chickens were matched on the collection year, with the purpose of having an equal number of MDR and pan-susceptible isolates in each year. The collection of 5 pairs of MDR and pan-susceptible *S. Typhimurium* isolates from retail chickens were examined for their genetic relationships. Metadata of selected isolates are provided (Table 2). CFSAN SNP Pipeline (Lite) (Davis et al., 2015) was used to conduct SNP-based cluster analysis and to examine the genetic relatedness of the isolates. FASTTREE (version 2.1.10) (Price et al., 2010) and Newick Display (version 1.6) (Dress et al., 2008) were used to construct the phylogenetic tree. Pairwise SNP differences were used to construct heatmap using Complex Heatmap package within R (Gu et al., 2016).

## Relationship between MDR isolates and other isolates in the NCBI Pathogen Detection

The relationship between five MDR isolates and other isolates from environment, food or human host were inferred by using the NCBI Pathogen Detection

<https://www.ncbi.nlm.nih.gov/pathogens/>. The NCBI Pathogen Detection clustered the MDR isolate genomes to other closely related isolates in the database via two steps: First, related isolates are clustered based on wgMLST scheme of *Salmonella* with a 25-allele cut-off; Once clusters are created, SNPs are called by aligning assemblies against a reference genome chosen from each cluster of closely related isolates, and SNP phylogenetic trees are inferred ([https://www.ncbi.nlm.nih.gov/pathogens/pathogens\\_help/](https://www.ncbi.nlm.nih.gov/pathogens/pathogens_help/)). Individual phylogenetic trees for each SNP clusters together with the metadata information of isolates in the same cluster were used to examine relationships within MDR clusters and pan-susceptible clusters.

## Results

### Phenotypic resistance of *S. Typhimurium* isolates

One hundred and fifty-three (26.5%) of the 577 clinical *S. Typhimurium* isolates were resistant to one or more classes of drugs and 104 (18.0%) exhibited resistance to three or more antimicrobial classes. *S. Typhimurium* isolates from retail meats were more resistant, with 87 (82.1%) of the 106 isolates being resistant to one or more antimicrobial classes and 43 (40.6%) being MDR. Among retail meat isolates, we observed that 50.0%, 25.0%, 22.2% and 12.5% of isolates from retail chickens, retail ground turkey, retail pork chops and retail ground beef were MDR, respectively. Although retail meat isolates were more resistant than clinical isolates for most drugs, they were less resistant than clinical isolates for streptomycin and chloramphenicol,

and showed no resistance to trimethoprim-sulfamethoxazole, nalidixic acid and ciprofloxacin (Table 2).

### **Correlation of genotypic and phenotypic resistance**

Six hundred and fifty-two (95.35%) of 683 isolates with complete phenotypic resistance data were included to examine the correlation between genotypic and phenotypic resistance. Overall, phenotypic resistance was highly correlated with predictive resistance based on the presence of known resistant genes. The overall concordance between phenotype and genotype was 99.63%, or 8,445 out of 8,476 tests (Table 3). The overall sensitivity was 96.47% and the overall specificity was 100.00% (Table 4). The disagreement (n=31) between the two methods were mostly related to streptomycin (n=16), but were also related to amoxicillin-clavulanic acid (n=1), sulfisoxazole (n=3), trimethoprim-sulfamethoxazole (n=1), ampicillin (n=3), chloramphenicol (n=1), ciprofloxacin (n=1), nalidixic acid (n=2), and tetracycline (n=3) (Table 4). For clinical isolates, sensitivity and specificity were 95.10% and 100.00%, respectively; for retail meat isolates, sensitivity and specificity were 98.55% and 100.00%, respectively (Table S1 & S2).

A total of 17 different resistant genes were identified from retail meat isolates (Figure 10) and the five most common ones were: *tet(A)* (n=90), *sul2* (n=79), *bla<sub>CMY-2</sub>* (n=39), *aph(3')-Ia* (n=7), *floR* (n=6). A total of 25 different resistant genes were identified from clinical isolates and the five most common ones were: *sul1* (n=54), *floR* (n=51), *sul2* (n=48), *aadA2* (n=41), *tet(A)* (n=41).



### **SNP analysis to infer relationship between MDR and pan-susceptible isolates**

The five MDR isolates differed by an average of 73 SNPs (range: 31-107 SNPs) from each other (Figure 11). In comparison, the five pan-susceptible isolates differed by an average of 473 SNPs (range: 142-618 SNPs). The nearest distance between a pan-susceptible and an MDR isolate was 547 SNPs (range: 547-657 SNPs). MDR isolates and pan-susceptible isolates distinctly clustered on a phylogenetic tree (Figure 12). Two MDR isolates (SRR6350864 and SRR7653324) that were separated by 65 SNPs and were collected in different states and years shared the same resistant profile and were originated from the same facility (P-667) (Table 2).

### **Relationship between MDR isolates and other isolates in the NCBI Pathogen Detection**

We accessed the NCBI Pathogen Detection on April 30, 2021, on which day there were 358,051 isolates of *Salmonella enterica* in the database. When relating the five MDR isolates with other isolates in the Pathogen Detection database, two SNP clusters were identified, i.e., [PDS000026710.292](https://www.ncbi.nlm.nih.gov/pathogens/tree#Salmonella/PDG000000002.2195/PDS000026710.292) (<https://www.ncbi.nlm.nih.gov/pathogens/tree#Salmonella/PDG000000002.2195/PDS000026710.292?term=PDS000026710.292>) and [PDS000030743.3](https://www.ncbi.nlm.nih.gov/pathogens/tree#Salmonella/PDG000000002.2195/PDS000030743.3) (<https://www.ncbi.nlm.nih.gov/pathogens/tree#Salmonella/PDG000000002.2195/PDS000030743.3?term=PDS000030743.3>). Note isolates that had an average SNP distance of 50 were clustered together in the Pathogen Detection. The SNP cluster PDS000026710.292 contained 1578 isolates with an average SNP distance of 51, and a minimum and maximum SNP distance of 0 and 105. Three MDR isolates from this study, i.e., SRR10857520, SRR9984357, and

SRR6350864 were included in this cluster. There were also 96 clinical isolates (6% of the total isolates) in this SNP cluster. Most of the remaining isolates in the cluster were collected from retail chicken samples in the U.S. The other SNP cluster PDS000030743.3 had 5 isolates with an average SNP distance of 23, and a minimum and maximum SNP distance of 1 and 36. One MDR isolate from this study, i.e., SRR8064311, one clinical isolate, and three other isolates from chicken samples were in this cluster. One MDR isolates from this study, SRR7653324, had no assigned cluster.

**Table 2** Metadata of *S. Typhimurium* isolates used for SNP analysis

<b>Isolate identifier</b>	<b>State</b>	<b>Date</b>	<b>Source</b>	<b>Establishment number</b>	<b>Accession number</b>	<b>Resistant pattern<sup>a</sup></b>	<b>Plasmids</b>
16MD05CB03	MD	5/9/2016	Chicken Breasts	P-667	SRR6350864	AMC, AMP, AXO, FIS, FOX, TET	IncA/C2, IncFIB(pHCM2)
16MD07CB32	MD	7/25/2016	Chicken Breasts	N/A	SRR6350796	n/a	IncFII(29)
17PA04CB18	PA	4/10/2017	Chicken Breasts	P-7903	SRR8064311	AMC, AMP, AXO, FIS, FOX, TET	IncA/C2
17MD02CB10	MD	2/13/2017	Chicken Breasts	P-806	SRR7907697	n/a	n/a
17PA08CB04	PA	8/14/2017	Chicken Breasts	P-667	SRR7653324	AMC, AMP, AXO, FIS, FOX, TET	IncA/C2
17TN11CB21	TN	11/9/2017	Chicken Legs	P705	SRR7907806	n/a	ColpVC, IncFIB(S), IncFII(S)
18GA12CB20	GA	12/27/2018	Chicken Breasts	P-9197	SRR9984357	AMC, AMP, AXO, FIS, FOX, TET	IncA/C2, IncX1
18NY02CB04	NY	02/05/2018	Chicken Breasts	P-1318	SRR9210855	n/a	IncFIB(S), IncFII(S), IncI1
18PA08CB35	PA	8/20/2018	Chicken Legs	N/A	SRR10857520	AMC, AMP, AXO, FIS, FOX, TET	IncA/C2
18WA08CB10	WA	8/3/2018	Chicken Thighs	P-6058	SRR9984387	n/a	n/a

<sup>a</sup>Antimicrobial agent abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AXO, ceftriaxone, FIS, sulfisoxazole; FOX, cefoxitin, TET, tetracycline.

**Table 3** Selected phenotypic resistance of *S. Typhimurium* isolates from humans and retail meats, United States 2016-2018

Source	N	Antimicrobial <sup>a</sup> n (%)								No. (%) of isolates resistant to multiple antimicrobial classes		
		AMC	AMP	AXO	CHL	COT	FIS	CIP	TET	≥1	≥3	≥5
Human	577	32 (5.55)	105 (18.20)	34 (5.89)	62 (10.75)	14 (2.43)	115 (19.93)	2 (0.35)	109 (18.89)	153 (26.52)	104 (18.02)	67 (11.61)
Retail Meats												
Retail Chickens	72	36 (50.00)	36 (50.00)	36 (50.00)	3 (4.17)	0	65 (90.28)	0	65 (90.28)	67 (93.06)	36 (50.00)	3 (4.17)
Retail Ground Beef	8	1 (12.50)	1 (12.50)	1 (12.50)	1 (12.50)	0	1 (12.50)	0	1 (12.50)	1 (12.50)	1 (12.50)	1 (12.50)
Retail Ground Turkey	18	3 (16.67)	4 (22.22)	3 (16.67)	0	0	14 (77.78)	0	15 (83.33)	16 (88.89)	4 (22.22)	1 (5.56)
Retail Pork Chops	8	1 (12.50)	3 (37.50)	1 (12.50)	2 (25.00)	0	3 (37.50)	0	2 (25.00)	3 (37.50)	2 (25.00)	1 (12.50)
Total Retail Meats	106	41 (38.68)	44 (41.51)	41 (38.68)	6 (5.66)	0	83 (78.30)	0	83 (78.30)	87 (82.08)	43 (40.57)	6 (5.66)
Total	683	73 (10.69)	149 (21.82)	72 (10.54)	68 (9.96)	14 (2.05)	198 (28.99)	2 (0.29)	192 (28.11)	240 (35.14)	147 (21.52)	73 (10.69)

<sup>a</sup>Antimicrobial agent abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AXO, ceftriaxone; CIP: ciprofloxacin; CHL, chloramphenicol; COT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; NAL, nalidixic acid; TET, tetracycline.

**Table 4** Comparison of genotypic and phenotypic resistance of *S. Typhimurium* isolates from humans and retail meats, 2016-2018

Antimicrobial <sup>a</sup>	Phenotypic resistant (n)		Phenotypic susceptible (n)		Sensitivity (%)	Specificity (%)
	Genotypic resistant	Genotypic susceptible	Genotypic resistant	Genotypic susceptible		
Aminoglycosides						
GEN	6	0	0	646	100.00	100.00
STR	90	16	0	546	84.91	100.00
β-Lactam/β-Lactamase Inhibitor						
AMC	66	1	0	585	98.51	100.00
Cephems						
FOX	66	0	0	586	100.00	100.00
AXO	66	0	0	586	100.00	100.00
Folate Pathway Inhibitors						
FIS	181	3	0	468	98.37	100.00
COT	7	1	0	644	87.50	100.00
Macrolides						
AZI	0	0	0	652	100.00	100.00

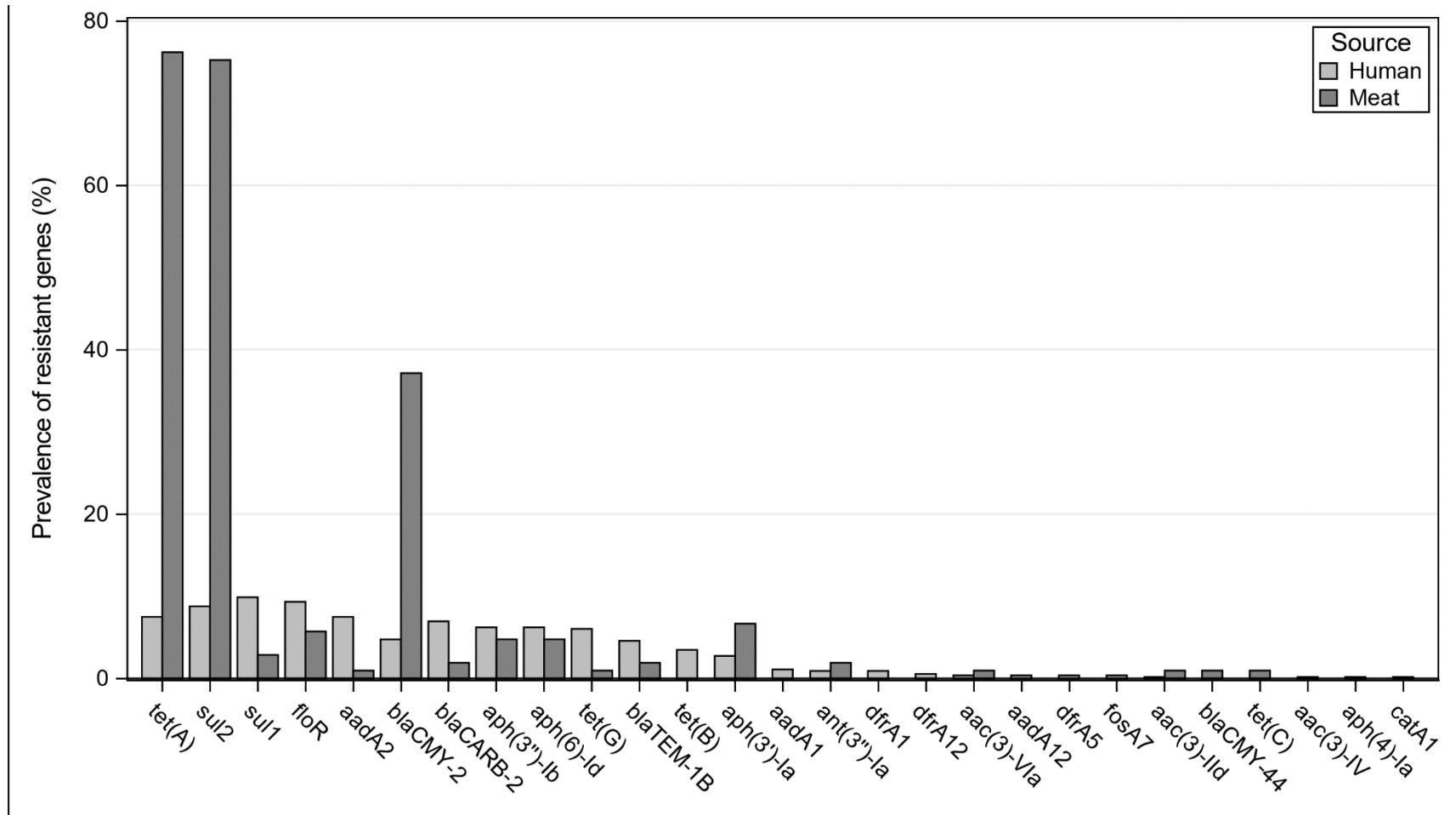
**Table 4** (continued)

Penicillins							
AMP	132	3	0	517	97.78	100.00	
Phenicol							
CHL	58	1	0	593	98.31	100.00	
Quinolones							
CIP	0	1	0	651	0.00	100.00	
NAL	0	2	0	650	0.00	100.00	
Tetracyclines							
TET	174	3	0	475	98.31	100.00	
Total	846	31	0	7,599	96.47	100.00	

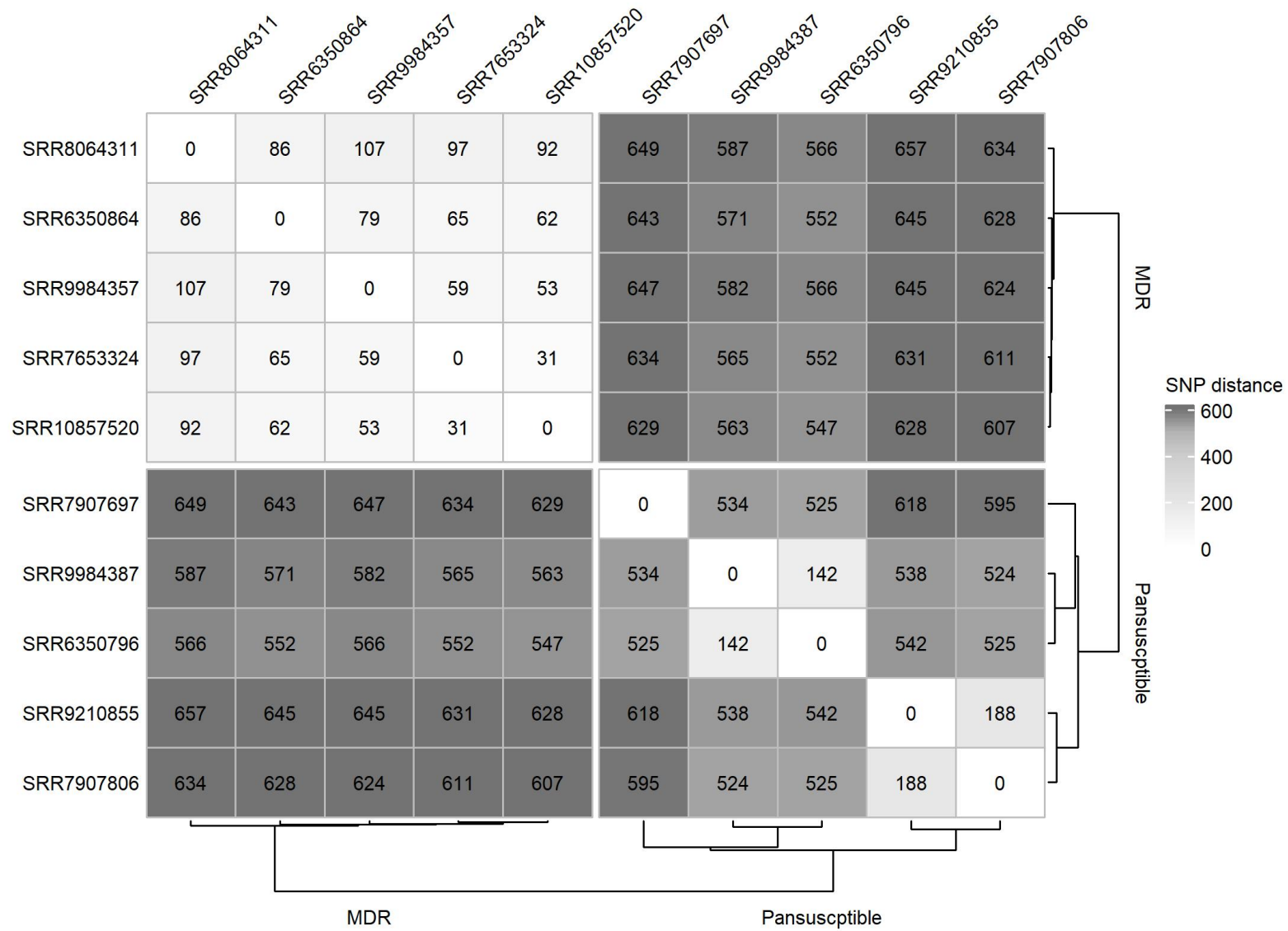
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<sup>a</sup>Antimicrobial agent abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AXO, ceftriaxone; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; COT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; FOX, ceftiofur; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline.

**Figure 10** Prevalence of resistance genes in *S. Typhimurium* by sources

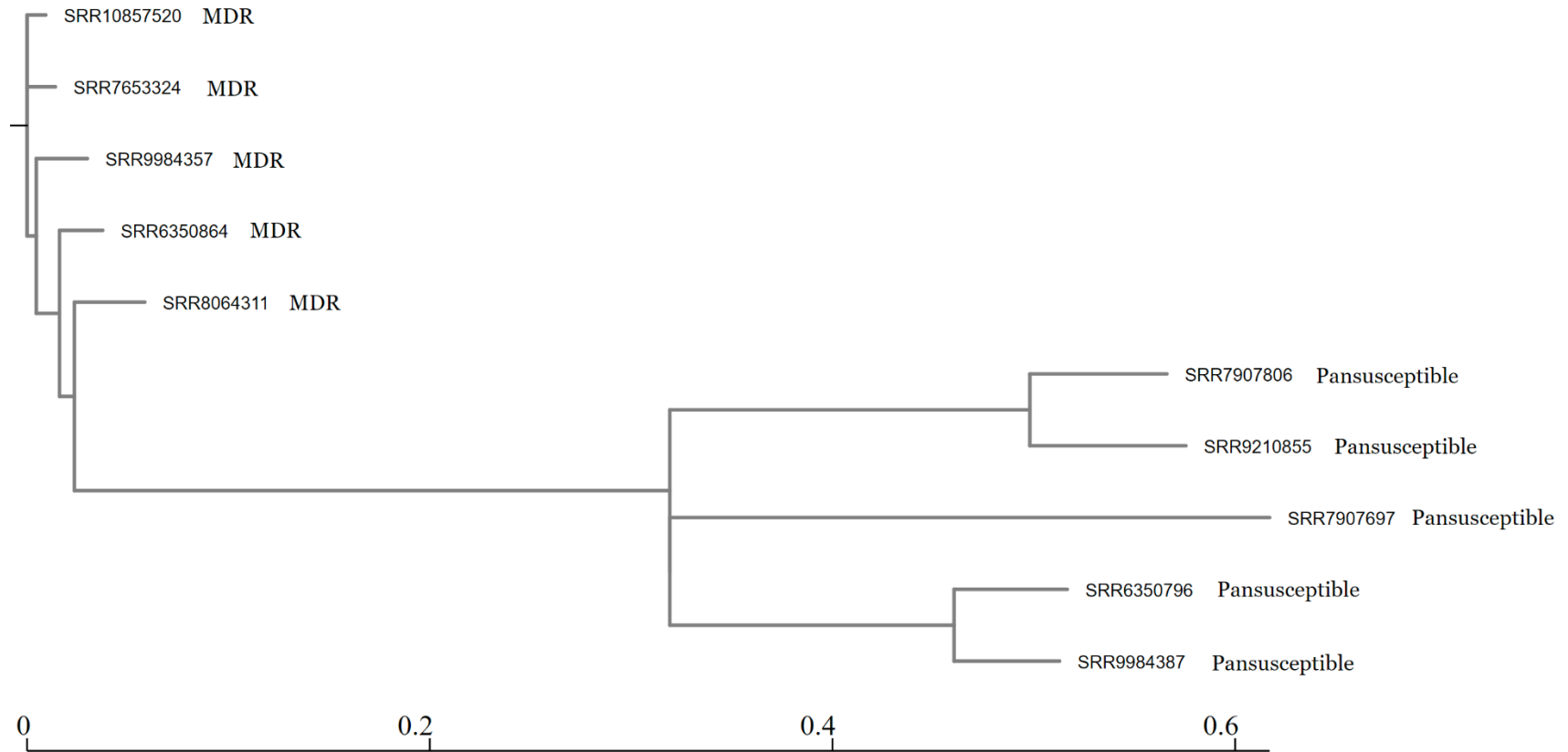


**Figure 11** Pairwise SNP distances between MDR and pan-susceptible isolates recovered from retail chicken samples





**Figure 12** Phylogenetic relationships of MDR and pan-susceptible isolates recovered from retail chicken samples



## Discussion

In this study, we analyzed *S. Typhimurium* isolates recovered from retail meats and humans during 2016-2018 using WGS. Our results suggested that MDR *S. Typhimurium* was more prevalent in retail meats than in humans, while the prevalence of chloramphenicol, trimethoprim-sulfamethoxazole and ciprofloxacin resistant isolates were higher in human isolates. The concordance between phenotypic and genotypic methods was very high, with the two methods agreeing on 99.63% of the antimicrobial resistance results. Comparative SNP analysis revealed that MDR *S. Typhimurium* are genetically closer compared to pan-susceptible *S. Typhimurium* isolates.

Overall, over 40% of *S. Typhimurium* isolates from retail meats were resistant to three or more antimicrobials, which was about twice as high as that in humans. The high prevalence of MDR *S. Typhimurium* in retail meats poses a public health concern, as the resistance may be transmitted to humans through the food chain and increase burden of illness (Aarestrup et al., 2014; Martin et al., 2004; Wang et al., 2019). The observed lower prevalence of MDR clinical isolates may be explained by the diverse sources of *S. Typhimurium* infections in humans. Other than consumption of retail meats, people can also be infected by cross contamination at home, through contact with other people and pets, as well as by consumption of vegetables and fruits. However, these other possible sources of *S. Typhimurium* infections are less likely to be exposed to antibiotics compared to consumption of retail meats. (Greene et al., 2008; Jackson et al., 2013; Varma et al., 2006). Antimicrobial treatment such as ciprofloxacin and ceftriaxone are indicated for patients with severe illnesses or at risk of invasive diseases (Shane et al., 2017). However, while resistance to quinolones like ciprofloxacin were low, around 40% of retail meats isolates

and 6% of human isolates were resistant to ceftriaxone, suggesting that quinolones should be considered as a better choice for clinical use and in animal husbandry.

In our study, the AMR phenotype is highly correlated with genotypic prediction of resistance, which is consistent with the results of previous epidemiological studies (McDermott et al., 2016; Nair et al., 2016; Neuert et al., 2018; Pornsukarom et al., 2018). A NARMS study (McDermott et al., 2016) reported 99% correlation between genotypic and phenotypic susceptibility after testing 640 *Salmonella* isolates from retail meats and humans. Similarly, a study in the UK (Neuert et al., 2018) with a larger dataset (n=3,491 NTS clinical isolates) identified 97.82% genotypic and phenotypic correlation. Another study in the UK focusing on testing *S. Typhimurium* from animals and plants observed 89.8% concordance (Mensah et al., 2019). Notably, most studies consistently pointed out that the discordance rates were mostly related to streptomycin. Since streptomycin is not used to treat enteric infections, precise clinical breakpoint was absent for testing susceptibility to this specific antimicrobial in NTS pathogens. High breakpoint for streptomycin resistance is one the main reasons that isolates carried resistance genes showing phenotypical susceptibility in prior studies (McDermott et al., 2016; Tyson et al., 2016). With the change of breakpoint from  $\geq 64$   $\mu\text{g/ml}$  to  $\geq 32$   $\mu\text{g/ml}$  in 2014, our study results showed that while the sensitivity of WGS dropped from 98% to 85%, the specificity increased from 91% to 100%. The mismatches of these two methods for other antimicrobials are negligible and may be due to reasons including silent resistance genes, mechanisms attenuating or inactivating gene expression, and the existence of unknown resistance genes may also contribute to the discordance (Adesiji et al., 2014; Garcia-Migura et al., 2012; Heider et al., 2009; Koskiniemi et al., 2011; Mensah et al., 2019).

Previous studies have used the high discriminatory power and accurate phylogenetic inferences of WGS and SNP-based analysis to improve outbreak investigation and examine genetic relationship between outbreak related isolates and sporadic isolates (Ashton et al., 2015; Keefer et al., 2019; Taylor et al., 2015). A study in Minnesota (Taylor et al., 2015) conducted SNP-based cluster analysis of 55 foodborne outbreak and sporadic *S. Enteritidis* isolates. The related outbreak isolates were found tightly clustered, with pairwise distance no more than 3 SNPs, while the nearest sporadic isolate to an outbreak isolate differed by an average of 42.4 SNPs. These findings suggest that WGS is an effective tool in identifying outbreak clusters and tracing their sources.

In this study, we focused on the genetic relationship between MDR and pan-susceptible *S. Typhimurium* isolates. SNP based phylogenetic analysis were used to examine the genetic relationship of five pairs of MDR and pan-susceptible isolates which were from the same source (retail chicken samples) and the same time period (2016-2018). We assumed that isolates from the same source type would have less variability, and a greater likelihood of similar or related strains, thereby providing better comparisons between MDR and pan-susceptible isolates. Retail chicken isolates were selected for SNP profiling because CDC NARMS data do not include specific sources of the infections among humans, which means clinical isolates may come from diverse sources include retail meats, vegetables and environmental sources (Greene et al., 2008; Jackson et al., 2013; Varma et al., 2006). We found that MDR and pan-susceptible *S. Typhimurium* isolates were distinctly clustered, and the within group SNP distance of MDR isolates (73.1) were closer compared to the within group distance of pan-susceptible isolates (473.1). Our results are consistent with a previous study conducted by researchers from Denmark

in that MDR isolates were genetically more uniform compared to pan-susceptible isolates (Leekitcharoenphon et al., 2016).

The presence of MDR in *S. Typhimurium* has been found to be linked to the acquisition of resistance genes harbored on mobile genetic elements such as plasmids, genomic islands, transposons and integrons, which are widely considered as tools for horizontal transfer (De Vito et al., 2015). For example, genomic island (SGI-1) was found attributable for the rapid evolution and transmission of MDR *S. Typhimurium* DT104 over the past few decades (Leekitcharoenphon et al., 2016; Paul et al., 2016). The dissemination of MDR *S. Typhimurium* through the food chain raises serious concern for public health, as it limited treatment options and undermined treatment outcomes for *Salmonella* infections.

All the five MDR isolates carried the IncA/C2 plasmid, while none the pan-susceptible isolates contain this specific plasmid. It is possible that all the AMR genes (*bla*<sub>CMY-2</sub>, *sul2*, *tet(A)*, *aph(3')-Ia*, and *bla*<sub>TEM-1B</sub>) may have been located on IncA/C2 plasmid (Table 2). The incompatibility group A/C (IncA/C) plasmids were first identified in 1970s from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and now found in a broad range of gram-negative bacteria such as *Escherichia coli*, *Salmonella enterica*, *Yersinia pestis*, etc. (Harmer & Hall, 2015). They were MDR plasmids that often carried the *bla*<sub>CMY-2</sub>, *sul2*, and *tet(A)* genes (Papagiannitsis et al., 2016). IncA/C2 also contained multiple resistance islands, which allowed them to acquire other resistance determinants and contribute to further plasmid evolution. When relating the five MDR isolates to other isolates in the Pathogen Detection database, the isolates in the same SNP clusters (i.e., SNP cluster PDS000026710.292 and SNP cluster PDS000030743.3) had a similar MDR pattern as the MDR isolates from this study, suggesting, the wide spread of the IncA/C2 plasmid among chicken-sourced *S. Typhimurium* in the U.S. Note clinical isolates

showing the same MDR pattern were found in these clusters, which may suggest the spread of these MDR isolates from chicken to human beings.

This study is subject to limitations. First, this study is focused on *S. Typhimurium* and the results may not be generalizable to other serotypes. Second, only ten isolates from retail chickens were selected for SNP analysis due to the limited availability of pan-susceptible isolates. Therefore, studies with larger sample size are warranted to estimate more accurate SNP distance between MDR and pan-susceptible isolates. Despite these limitations, this retrospective study has provided evidence for the value and reliability of WGS in predicting serotype specific antibiotic resistance, and provided information for better understanding the evolution of MDR *S. Typhimurium* isolates. When data are available, they can be used to explore global relatedness of *S. Typhimurium* isolates.

## Chapter 5: Conclusions

Previous studies suggest that the level of AMR pathogens in animal production is positively associated with antimicrobial use in that population (Chantziaras et al., 2014; McEwen et al., 2002; Roth et al., 2019; Tang et al., 2017). However, little has been done to quantify their association and to understand the genetic mechanism of MDR NTS recovered from retail meats. The present two studies used a subset of the publicly available NARMS database mainly to: 1) examine the associations of fluoroquinolones use in food animals with quinolone resistance in NTS from retail meats; 2) examine the genetic relatedness of MDR and pan susceptible NTS isolates with WGS method.

We observed that the prevalence of quinolone resistant NTS from retail meats increased from 0% in 2013 to 12.75% in 2018. During the same time, the sales of fluoroquinolones in food animals increased from 15,099 kg to 23,350 kg, indicating a positive correlation between the prevalence of quinolone resistant NTS in retail meats and fluoroquinolone sales in food animals ( $r=0.67$ ). Our results also showed a high concordance between phenotypic and genotypic methods in detecting AMR among NTS isolates (99.63%), and SNP analysis revealed that MDR *S. Typhimurium* isolates were genetically closer compared to pan susceptible isolates.

Combined with previous literatures explaining potential biological mechanisms of antimicrobial use in veterinary medicines and subsequent resistance (Angulo et al., 2000; Gupta et al., 2004; McEwen et al., 2002; Piddock, 2002; Richard et al., 1994), our results supported that positive association and further provided evidence that injudicious use of antimicrobials in food animals may contribute to the emergence of antimicrobial resistance. Moreover, our study provided evidence for the reliability of WGS in predicting antibiotic resistance of NTS, and explored ways of using WGS to better understand the evolution of MDR NTS.

Further study with actual antimicrobial use in food animals and longer time frame may be helpful in understanding the lag between antimicrobial use and subsequent resistance, the lingering time of resistance after the stop of drug use, and to better quantify their association. Also, additional studies with larger sample size and long sequence reads of NTS isolates from various sources can aid to explain the closeness of MDR NTS isolates compared to pan-susceptible ones.

Altogether, the findings from current studies signify the importance of sustained surveillance of NTS and other foodborne pathogens in humans, retail meats, food animals, and the environment. Multi-sectoral and cross-disciplinary efforts following the “One Health approach” are needed to reduce the spread of AMR in both humans and animals.



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## Appendix

**Supplemental table 1** Comparison of genotypic and phenotypic resistance of *S. Typhimurium* isolates from retail meats, 2016-2018

Antimicrobial <sup>a</sup>	Phenotypic resistant (n)		Phenotypic susceptible (n)		Sensitivity (%)	Specificity (%)
	Genotypic resistant	Genotypic susceptible	Genotypic resistant	Genotypic susceptible		
Aminoglycosides						
GEN	2	0	0	103	100.00	100.00
STR	8	3	0	94	72.73	100.00
β-Lactam/β-Lactamase Inhibitor						
AMC	40	0	0	65	100.00	100.00
Cephems						
FOX	40	0	0	65	100.00	100.00
AXO	40	0	0	65	100.00	100.00
Folate Pathway Inhibitors						
FIS	81	1	0	23	98.78	100.00
COT	0	0	0	105	100.00	100.00

**Supplemental table 1** (continued)

Macrolides							
AZI	0	0	0	105	100.00	100.00	
Penicillins							
AMP	43	0	0	62	100.00	100.00	
Phenicol							
CHL	6	0	0	99	100.00	100.00	
Quinolones							
CIP	0	0	0	105	100.00	100.00	
NAL	0	0	0	105	100.00	100.00	
Tetracyclines							
TET	81	1	0	23	98.78	100.00	
Total	341	5	0	1,019	98.55	100.00	

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<sup>a</sup>Antimicrobial agent abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AXO, ceftriaxone; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; COT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline.

**Supplemental table 2** Comparison of genotypic and phenotypic resistance of *S. Typhimurium* isolates from humans, 2016-2018

Antimicrobial <sup>a</sup>	Phenotypic resistant (n)		Phenotypic susceptible (n)		Sensitivity (%)	Specificity (%)
	Genotypic resistant	Genotypic susceptible	Genotypic resistant	Genotypic susceptible		
Aminoglycosides						
GEN	4	0	0	543	100.00	100.00
STR	82	13	0	452	86.32	100.00
β-Lactam/β-Lactamase Inhibitor						
AMC	26	1	0	520	96.30	100.00
Cephems						
FOX	26	0	0	521	100.00	100.00
AXO	26	0	0	521	100.00	100.00
Folate Pathway Inhibitors						
FIS	100	2	0	445	98.04	100.00
COT	7	1	0	539	87.50	100.00
Macrolides						
AZI	0	0	0	547	100.00	100.00

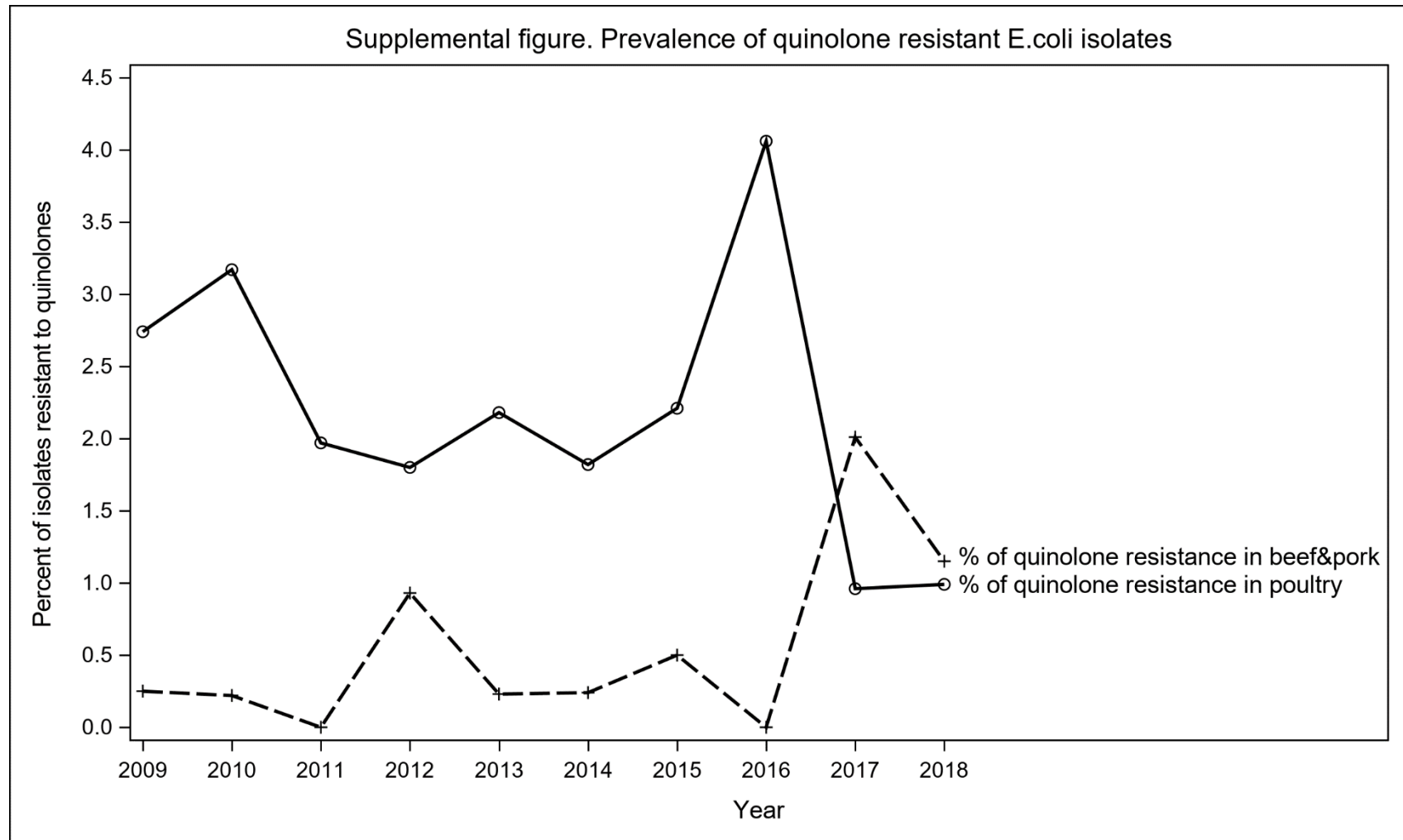
**Supplemental table 2** (continued)

Penicillins							
AMP	89	3	0	455	96.74	100.00	
Phenicol							
CHL	52	1	0	494	98.11	100.00	
Quinolones							
CIP	0	1	0	546	0.00	100.00	
NAL	0	2	0	545	0.00	100.00	
Tetracyclines							
TET	93	2	0	452	97.89	100.00	
Total	505	26	0	6,580	95.10	100.00	

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<sup>a</sup>Antimicrobial agent abbreviations: AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AXO, ceftriaxone; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; COT, trimethoprim-sulfamethoxazole; FIS, sulfisoxazole; FOX, cefoxitin; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline.

**Supplemental figure** Prevalence of quinolone resistant *E.coli* isolates





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## EDUCATION

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<b>College of Medicine, Penn State University</b>	Hershey, PA
<i>Doctor of Public Health in Epidemiology and Biostatistics</i>	Current GPA: 3.78/4.00
	Aug 2018 –Present
<b>Rollins School of Public Health, Emory University</b>	Atlanta, GA
<i>Master of Public Health in Epidemiology</i>	Cumulative GPA: 3.74/4.00
	Aug 2015 –Dec 2017

## PROFESSIONAL EXPERIENCE

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<b>College of Medicine, Penn State University</b>	Hershey, PA
<i>Research Assistant</i>	Dec 2019 – Present
<ul style="list-style-type: none"><li>Co-developed an agent-based microsimulation model using Python, aimed at assessing the impact of different school reopening methods on community spread of COVID-19</li><li>Developed an interactive web tool using R Shiny to allow our simulation model users to input their own parameters</li><li>Conducted data analysis of assessing the effect of telementoring program on improving HCV care using Medicare data, and co-authored an article (under review)</li><li>Conducted data analysis of examining the association of DAA treatment with mortality among patients with HIV/HCV co-infection using Medicare data, and co-authored an article (under review)</li></ul>	
<b>Bureau of Epidemiology, Pennsylvania Department of Health</b>	Harrisburg, PA
<i>NARMS Intern</i>	Aug 2018 – Present
<ul style="list-style-type: none"><li>Analyzed FDA NARMS retail meat isolates database to characterize antimicrobial resistance in <i>Salmonella</i> recovered from retail poultry by antibiotic claims; presented study results (poster and oral) on IDweek 2019 and 35<sup>th</sup> ICPE; co-authored an article (under review)</li><li>Conducted a study to examine the extent to which public health jurisdictions are disseminating surveillance findings to promote judicious use of antimicrobials; co-authored an abstract (accepted by IDweek 2020)</li><li>Analyzed genomic data using GalaxyTrakr to determine serotype, resistant genes and predictive resistant antimicrobial of <i>Salmonella</i> isolates</li></ul>	
<b>Centers for Disease Control and Prevention</b>	Atlanta, GA
<i>ORISE Fellow</i>	Dec 2017– May 2018
<ul style="list-style-type: none"><li>Designed an analysis to examine the association between proficiency testing failure and future Clinical Laboratory Improvement Amendments (CLIA) deficiencies using SAS and R; presented study results on the 2nd Annual CDC Laboratory Science Symposium (poster and oral)</li><li>Conducted data analysis of examining the association of opioid use with chronic pain status using Medicare data</li></ul>	
<b>Rollins School of Public Health, Emory University</b>	Atlanta, GA
<i>Research Assistant &amp; Teaching Assistant</i>	Sep 2016 – Dec 2017
<ul style="list-style-type: none"><li>Designed a geospatial analysis of opioid spending in the U.S. using SAS</li><li>Conducted analysis to examine the effect of a new treatment on brain damage recovery using data from Grady hospital</li><li>Led group discussions, graded student assignments and held office hours for the course <i>Health Economics</i></li></ul>	
<b>The Task Force for Global Health</b>	Atlanta, GA
<i>GSK Fellow</i>	Jun 2016 – Aug 2016
<ul style="list-style-type: none"><li>Provided logistic support in planning and management of vaccine distribution to target developing countries</li><li>Provided editorial and research for publication on lessons in polio eradication</li></ul>	

## PUBLICATIONS

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- Yin, X., M'ikanatha, N.M., Nyirabahizi, E., McDermott, P.F. & Tate, H. (2021). Antimicrobial resistance in non-Typhoidal *Salmonella* from retail poultry meat by antibiotic usage-related production claims - United States, 2008-2017. *Int J Food Microbiol*, 342:109044. doi: 10.1016/j.ijfoodmicro.2021.109044. Epub 2021 Jan 5. PMID: 33529874.
  - Yin, X. & Bostick, RM. (2019). Associations of Nut Intakes with Incident Sporadic Colorectal Adenoma: A Pooled Case-Control Study. *Nutr Cancer*, 71(5): 731-738. doi: 10.1080/01635581.2018.1521440.

## LANGUAGES & SKILLS

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- Computer Skills:** R, Python, SAS, R Shiny, ArcGIS Pro, SQL, Tableau, SEER\*Stat, HTML&CSS, TreeAge, Office