EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) AND NON-TYPHOIDAL *SALMONELLA* (NTS) IN MICHIGAN

By

Sanjana Mukherjee

A DISSERTATION

Submitted to
Michigan State University
in partial fulfilment of the requirements
for the degree of

Microbiology and Molecular Genetics - Doctor of Philosophy

2018

ABSTRACT

EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC) AND NON-TYPHOIDAL *SALMONELLA* (NTS) IN MICHIGAN

By

Sanjana Mukherjee

The enteric pathogens, Shiga toxin-producing *E. coli* (STEC) and non-typhoidal *Salmonella* (NTS), are leading causes of foodborne infections in the US, resulting in 265,000 and 1.2 million illnesses every year, respectively. The emergence of antibiotic resistance in these pathogens has been documented and is of great concern due to negative patient health outcomes and the possibility of transfer of resistance genes to other clinically relevant pathogens. However, there is a scarcity in information about frequencies of antibiotic resistant and factors associated with resistant STEC and NTS infections in Michigan. It is necessary to have a complete understanding about the of emerging antibiotic resistance and factors driving the rise of resistance in STEC and NTS to help develop effective control strategies. In this dissertation, 980 STEC isolates collected from patients in Michigan between 2001 and 2014 were examined for resistance to clinically relevant antibiotics. The examination of STEC strains for resistance, revealed high frequencies of resistance to ampicillin and trimethoprim-sulfamethoxazole, with significant increases in antibiotic resistance rates observed over this 14-year period. Multivariate logistic regression analysis identified non-O157 serotypes to be independently associated with antibiotic resistance. The recent increase in incidence of non-O157 serotypes observed in the US, coupled with the high frequencies of antibiotic resistance observed in this study, suggest the emergence of antibiotic resistant non-O157s as important human pathogens. Additionally, antibiotic resistant STEC isolates from patients in recent years (2010-2014) were more likely to cause hospitalizations

than pansusceptible STEC isolates, suggesting that resistant STEC infections may result in adverse patient outcomes. Using whole genome sequencing, we also identified chromosomal mutations and 33 horizontally acquired genes present in the genomes of non-O157 STEC, likely conferring resistance. Importantly, by creating a co-occurrence network of these genes, we identified the co-occurrence of certain resistance genes, which are possibly present on the same mobile genetic element, thus resulting in multi-drug resistance. In addition to examining resistance in STEC, a total of 198 clinical NTS isolates collected between 2011 and 2014 were also examined for antibiotic resistance in this dissertation. Resistance to tetracycline, trimethoprim-sulfamethoxazole and ampicillin were commonly observed. Concerningly, high frequencies of multidrug resistant NTS were also observed with significant increases in their prevalence observed between 2011 and 2014. These high multidrug resistant rates have important implications on patient care as the efficacy of multiple antibiotics is reduced. Antibiotic resistant NTS isolates were also found to result in significantly longer mean hospital stays compared to pansusceptible NTS. Serovar specific differences in frequencies of antibiotic resistance were observed; S. Enteritidis were observed to have lower resistance frequencies than other serovars. Lastly, to better understand the role that cattle reservoirs play in harbouring antibiotic resistant STEC strains, we examined 121 STEC isolates collected in 2012 from six cattle farms in Michigan for antibiotic resistance. While high resistance frequencies to tetracycline and trimethoprim-sulfamethoxazole were observed in certain herds, no resistance to ampicillin was observed, unlike what was observed in STEC isolates collected from patients. While different populations of resistant STEC may be circulating in the clinical and agricultural environments, continuous monitoring of resistance in the cattle reservoir is warranted to determine if animal reservoirs can serve as potential sources of resistant infections in humans.

For my grandparents, Dadu and Dia

ACKNOWLEDGEMENTS

"It takes a village to raise a scientist". I recently came across the saying when I attended a seminar presentation by Dr. Vincent Young and I realized that it was the perfect opening line for my acknowledgements page. I have so many people to thank and acknowledge for helping me throughout my journey in obtaining a doctorate degree. First, I would like to thank my mentor, Dr. Shannon Manning, who has been an incredible mentor throughout my graduate career. Although I did not have any prior training in Epidemiology when I first joined MSU, she took a chance on me and welcomed me into her lab. Working under her guidance has made me into a better scholar and I continue to be inspired by her research capabilities every day. Not only has she been an incredible research mentor, but her warmth and friendliness made the Manning Lab feel like my second home and I feel incredibly lucky that I had the opportunity to be mentored by her.

I would also like to thank my committee members Dr. Linda Mansfield, Dr. Bo Norby and Dr. Lixin Zhang for their expert guidance, valuable advice and words of encouragement. While Dr. Mansfield and Dr. Norby helped improve my knowledge on the veterinary components of my research, Dr. Zhang's expertise in Infectious Disease Epidemiology helped polish my scientific concepts.

I am incredibly grateful to have had an opportunity to work alongside all the members of the Manning Lab. I started my journey in the Manning Lab by working closely with Pallavi Singh, Rebekah Mosci and Wonhee Cha. Pallavi help me understand the basics of sequencing, Rebekah organized the epidemiological data and Wonhee taught me the basics of SAS coding and epidemiological study designs. In addition, Samantha Wengert was instrumental in identifying the molecular profiles of STEC isolates from cattle. I would also like to thank Heather Blankenship

and Brian Nohomovich for their help with the sequencing and coding aspects of my project. Chase Anderson played an important role in the completion of this project by helping with antibiotic susceptibility testing and Brian Snyder helped with the molecular typing of STEC isolates. I am also incredibly grateful for the advice and friendship of other lab members, present and past, including Cole McCutcheon, Megan Shiroda, Jose Rodrigues, Zoe Hansen, Macy Pell, Rebecca Flaherty, Robert Parker, Michelle Korir, Katherine Jernigan, Maja Magel, Matt Peters, Joe Bednarczyk, Jessica Plemmons, Clare Laut, David Knupp, Moriah Moore and Bridget Walker. I have made so many incredible memories with this group of people, some of which include 'Manning Lab Prank Wars', the Philly road trip, the Grad Office shenanigans, the Chick-fil-A outings, my very own "Sanjana" buttons for my dissertation defense and the end of the year holiday parties. Not only are they the greatest co-workers but they are also the greatest friends.

Financial support for this work came from National Institute of Health, Enteric Research Investigational Network (grant # U19AI090872), U.S. Department of Agriculture, National Institute of Food and Agriculture (grant # 2011-67005-30004), Bertina Wentworth Scholar Award, College of Natural Science Dissertation Continuation Fellowship and the Ronald and Sharon Rogowski Fellowship from Michigan State University. I would also like to thank Dr. James Rudrik, James Collins and the staff of the Michigan Department of Human Health and Services for their support in the completion of this project. In addition, I would also like to thank the faculty, graduate students, post-doctoral researchers and the staff of the Department of Microbiology and Molecular Genetics for their scientific advice, words of encouragement and help.

Lastly, I would also like to thank my family and friends at MSU, back home in India and those scattered around the globe for cheering me on throughout this entire process. Gizem Kurt and Sahra Uygun were my first friends here at MSU, and our countless visits to the Wharton

Theatre and haunted houses made my years at MSU extremely memorable. My mother, my best friend Adwiti, my cousin Imara, my grandmother, my brother Sheel, my uncle, my aunt and my cousin Avik have always taken an active interest in my research and have been my biggest cheerleaders while I set out to complete my doctoral degree.

It truly does take a village to raise a scientist.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ACSSuT	Ampicillin, Chloramphenicol, Streptomycin, Sulfisoxazole and Tetracycline
aEPEC	Atypical Enteropathogenic *E. coli*
AMP	Ampicillin
ARG	Antibiotic Resistance Genes
ATCC	American Type Culture Collection
CARD	Comprehensive Antibiotic Resistance Database
CARSS	Canadian Antimicrobial Resistance Surveillance System
CDC	Centers for Disease Control and Prevention
CHRT	Center for Healthcare Research and Transformation
CI	Confidence Interval
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
DALY	Disability Adjusted Life Years
EARS-Net	European Antimicrobial Resistance Surveillance Network
EcMLST	*E. coli* Multi Locus Sequence Typing
EHEC	Enterohemorrhagic *E. coli*
ERIN	Enterics Research Investigational Network
ESBL	Extended Spectrum β-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FoodNet	Foodborne Disease Active Surveillance Network
GLASS	Global Antimicrobial Resistance Surveillance System
HUS	Hemolytic Uremic Syndrome

LEE	Locus of Enterocyte Effacement
MDHHS	Michigan Department of Health and Human Services
MDR	Multi-Drug Resistance
MDSS	Michigan Disease Surveillance System
MIC	Minimum Inhibitory Concentration
MLST	Multi Locus Sequence Typing
NARMS	National Antimicrobial Resistance Monitoring System
NCHS	National Center for Health Statistics
NGS	Next Generation Sequencing
NNDSS	National Notifiable Diseases Surveillance System
NPV	Negative Predictive Value
NTS	Non-Typhoidal *Salmonella*
OR	Odds Ratio
OTC	Over-The-Counter-Prescription
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
PPV	Positive Predictive Value
PT	Phage Type
QC	Quality Control
SG	SNP Genotype
SNP	Single Nucleotide Polymorphisms
ST	Sequence Type
STEC	Shiga Toxin-Producing *E. coli*
SXT	Trimethoprim-Sulfamethoxazole
TET	Tetracycline

USDA	United States Department of Agriculture
VFD	Veterinary Feed Directive
VTEC	Verotoxin producing *E. coli*
WGS	Whole Genome Sequencing
WHO	World Health Organization

CHAPTER 1

Literature Review: Epidemiology of Antibiotic Resistant Shiga Toxin-Producing Escherichia coli (STEC) and Non-Typhoidal Salmonella (NTS)

INTRODUCTION: THE BURDEN OF ENTERIC PATHOGENS

Diarrheal diseases are a global public health concern resulting in significant morbidity and mortality. The World Health Organization (WHO) estimates that diarrheal diseases are one of the leading causes of deaths worldwide, and in 2004, resulted in 2.2 million deaths (1). They are particularly problematic in low income countries, accounting for 6.9% of total deaths (1). In addition, diarrheal diseases are responsible for more than 1.5 million deaths in children less than five years of age (1). In 2010 alone, foodborne hazards contributed to approximately 600 million cases of food illnesses globally, of which 550 million have been attributed to infectious agents (2). While Norovirus and *Campylobacter* spp. were the leading cause of enteric illnesses among all infectious agents, non-typhoidal *Salmonella* (NTS) spp. and Shiga toxin-producing *E. coli* (STEC) resulted in approximately 78 million and 1 million cases, respectively (2). In addition, food illnesses caused by infectious agents can have a long-term effect on human health resulting in more than 17 million Disability Adjusted Life Years (DALYs); NTS resulted in 4 million DALYs, while STEC contributed to 13,000 DALYs (2). Although the burden of foodborne illnesses in North America is low compared to the rest of the world, diarrheal diseases have been estimated to cause 67% of the total health burden, with NTS and *Campylobacter* infections predominating (2). Food illnesses are costly, with substantial losses in the food industry and public health sector as well as in individual households. In the United States alone, the annual cost of illness by foodborne pathogens has been estimated to be $14 billion (3). NTS infections were estimated to cost the most when compared to all other foodborne pathogens with an annual cost of illness of $3.3 billion, while STEC infections result in $278 million in losses per year (3, 4). The FoodNet surveillance network was established by the Centers for Disease Control and Prevention (CDC), the United States Department of Agriculture (USDA), the Food and Drug Administration (FDA) and 10 state

health departments in 1995 (5). This active surveillance system collects samples from California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New Mexico, New York, Oregon and Tennessee, and thus, monitors the incidence of foodborne illnesses and collect case information associated with these illnesses in the US.

Shiga toxin-producing *Escherichia coli* (STEC)

Shiga toxin-producing *Escherichia coli* (STEC) is a gram-negative pathogen and major cause of foodborne infections worldwide. The most well-known member of this group of pathogenic *E. coli* is *E. coli* O157:H7, which was first identified as a pathogen in 1982 when an outbreak occurred in the states of Oregon and Michigan (6). Indeed, more than 472 serotypes have been identified (7), which vary based on the antigenic structure of the O antigen comprising the polysaccharide component of the cell wall lipopolysaccharide (LPS), and the flagellar H antigen. In the US, infections caused by serogroup O157 have been more commonly reported than all other serogroups (non-O157 serogroups) (8); however, infections caused by the O26, O103, O111, O121, O45 and O145 serogroups are increasing (9-11). According to the FoodNet surveillance system in the US, the overall incidence of O157 and non-O157 STEC infections, in 2015, was 0.95 and 1.65 per 100,000, respectively (12).

Pathogenesis and clinical presentation in humans

STEC have a very low infectious dose, i.e. <100 cells, that makes it a very potent pathogen (13, 14). In humans, symptoms of STEC infections usually presents 3-8 days after ingestion and results in watery diarrhea, abdominal pain, nausea and vomiting. In severe cases, STEC results in hemorrhagic colitis characterized by bloody diarrhea. Hemolytic uremic syndrome (HUS) is another severe complication resulting in thrombotic microangiopathy, thrombocytopenia and acute

kidney damage (15). Approximately 90% of HUS cases in children are attributed to STEC infections (15, 16). Although the O157 serotypes of STEC are frequently responsible for causing HUS (17-19), HUS cases caused by non-O157 serotypes have also been reported (18, 20).

STEC are characterized by their ability to produce the Shiga toxin (Stx), a cytotoxin that resembles the *Shigella dysenteriae* Type I toxin (21-23). Shiga toxins, the primary virulence factors of STEC, are AB_5 toxins with one enzymatically active subunit (A subunit) and five identical binding subunits (B subunit) (24). This B subunit binds to the glycosphingolipid receptor, globotriaosylceramide (Gb3), present on host renal cells (25, 26), triggering the inhibition of host protein synthesis via inactivation of the 60S eukaryotic ribosomal subunit (27). After binding to the Gb3 receptor via the B subunits, the toxin is endocytosed and trafficked retrograde through the Golgi apparatus to the endoplasmic reticulum. During this trafficking, the A subunit is proteolytically cleaved into the A1 and A2 fragments; the A1 fragment, which has N-glycosidase activity, is responsible for the depurination of the 28S rRNA resulting in inhibition of protein synthesis and thus, cell death (28-30). Consequently, the cell death of renal endothelial cells results in thrombotic microangiopathy, or thrombosis of renal capillaries, causing hemolysis of red blood cells and decreased glomerular perfusion leading to HUS (31, 32).

In 1977 Konowalchuk et al. reported the production of a cytotoxin by some *E. coli* strains which was cytotoxic towards Vero cells (28, 33). Subsequently in 1983, O'Brien et al. reported the production of Shiga like toxins by Enterohemorrhagic *E. coli* (EHEC) exhibiting cytotoxicity towards Vero cells (34). Due to the cytopathic effect of Shiga toxins on Vero cells, STEC are also referred to as Verotoxin producing *E. coli* (VTEC) (35). There are two different types of the STEC Shiga toxins, Stx1 and Stx2, which are genetically related but antigenically distinct (28, 36). While the Stx1 is >99% homologous to the *Shigella dysenteriae* toxin, Stx2 shares only 50-60% amino

acid homology with Stx1 (37-39). Shiga toxins may cross the human intestinal epithelium via Gb-3 independent transcytosis, translocation by neutrophil transmigration, Gb-3 dependent movement by Paneth cells and transcytosis by M cells (40, 41). However, Stx1 and Stx2 may cross the intestinal epithelium differently as significantly lower levels of Stx2 were observed to move across polarized intestinal epithelial cells than Stx1 (42). The *stx* genes are carried by Stx-encoding bacteriophages (43, 44) present in the late gene region of lysogenic lambdoid phages (45). The shiga toxin genes are expressed when the lytic cycle of the phage are activated (46-48) which is usually a result activation of the bacterial SOS response (49). The circulation of Stx-encoding bacteriophages in the environment has been observed; the presence of free *stx* phages has been reported in sewage, waste water, river water and in cattle feces (50-52). The dissemination of *stx* bacteriophages in the environment is concerning as it could result in the evolution of novel pathogens. Indeed, the *E. coli* O104:H4 strain acquired a prophage encoding the Shiga toxin 2a variant and was responsible for a large outbreak in Germany in 2011 (53, 54).

In addition to the Shiga toxin, the locus of enterocyte effacement (LEE) pathogenicity island plays an important role in the pathogenesis of STEC infection. The 35.5-kb LEE pathogenicity island encodes for the intimin protein (*eae* gene) and the translocated intimin receptor (Tir) which are crucial for the development of attaching and effacing (A/E) lesions on intestinal mucosa (14, 55-57). In addition, the LEE pathogenicity island also encodes a type III secretion system which is responsible for secreting proteins into the host cell cytoplasm (14). Other virulence factors include plasmid-encoded enterohemolysin (*ehx*) (58) and an autoagglutinating adhesin (Saa) (59). Additionally, STEC must also survive the acidic pH in the stomach which it does so with the help of acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system (60-63). Bacterial attachment to the enterocytes via

proteins produced by the organism is the first step in causing infections. The binding of the intimin protein to the Tir receptor, which is inserted into the host cell membrane, results in the formation of 'pedestals' thus resulting in histological changes and accumulation of actin in the enterocytes. The intestinal colonization and subsequent Shiga toxin production results in the disease (64).

Reservoirs of STEC and its transmission to humans

STEC have been frequently isolated from many animals such as cattle, sheep, pigs and goats (65-67); however, cattle are considered a primary reservoir worldwide (67-71). A study of STEC in dairy farms in Wisconsin and Washington, for example, observed that 8% of adult cows and 19% of the calves and heifers were positive for shedding STEC (72). Additionally, Cernicchiaro et al. reported the isolation of STEC in beef cow-calf farms in Ontario, with 45% of total farms testing positive for *E. coli* O157:H7 (73). A recent study in Michigan, looking at six dairy herds and five beef herds, reported a higher prevalence of STEC in beef cattle (21%) than in dairy cattle (13%) (74).

Fecal shedding of STEC by cattle is an important source of transmission of STEC in the environment, and some cattle are referred to as 'super shedders' if they excrete more than 10^3 - 10^4 colony forming units (CFU) of STEC per gram of feces (75). Thus, studies have attempted to identify factors associated with STEC shedding in cattle to guide the development of new strategies that can reduce the transmission of STEC (74, 76). Cattle are asymptomatic carriers of STEC, which have been isolated from the rectum, rumen and colon (14, 77-79). Grauke et al. also identified the lower gastrointestinal tract of ruminants to be the predominant site of *E. coli* O157:H7 proliferation (80). The lack of Gb3 receptors in the gastrointestinal tract of cattle

provides insights into why cattle are asymptomatic carriers of STEC (81). However, STEC have been implicated as causative agents of diarrhea and dysentry in calves (82).

Transmission of STEC to humans occurs via multiple infection routes, which include contact with animal reservoirs, consumption of contaminated food and water, and by person-person contact, or secondary transmission. Animal contact is an important source of domestically acquired STEC infections in humans, causing an estimated 6% of STEC O157 and 8% of non-O157 infections (83). Indeed, a recent study in the Netherlands and other European countries identified animals to be important sources of human STEC infections; 48.6% of total human cases were attributed to cattle alone (84). Petting zoos have also been identified as sources of STEC infections (85) and have been implicated in multiple outbreaks (86, 87). Consumption of contaminated beef products (88, 89), especially undercooked beef, is an important source of human STEC infections. The CDC, for instance, has linked consumption of contaminated beef products to several multistate STEC outbreaks in the US, which include the 2016 and 2014 *E. coli* O157:H7 outbreaks resulting in high hospitalization rates and product recalls (90). Other contaminated food products such as raw milk (91-93), fresh produce (94, 95) and fermented sausages and salami (96, 97), have also contributed to STEC infections in humans. Water contaminated with STEC is also an important source of infection and transmission vehicle (98, 99). Fruits and vegetables can be contaminated by STEC via irrigation water that carries STEC from contaminated feces found in agricultural settings (100, 101). Strachan et al. predicted that contact with the environment, which includes contact with animals, their feces and contaminated water, caused 54% of STEC outbreaks in Scotland (102). Person-person contact of STEC infections also plays a critical role in the spread of STEC infections in the community (103), which is likely due to the low infectious dose.

Risk factors of STEC infections

The most frequently reported risk factor of STEC infection is the consumption of contaminated beef (104-107). Since cattle is an important reservoir of STEC, these pathogens have been commonly isolated from ground beef (108-110) and many outbreaks in the US have been attributed to contaminated beef (111). Another important risk factor is direct or indirect contact with animals. Indeed, a matched case-control study looking at risk factors of STEC O157 infections in the US identified farm visits as an important risk factor; while living on a farm or farm visits were risk factors for persons below and above six years of age, contact with cows was identified to be a risk factor for persons more than six years of age (106). These findings are consistent with several prior studies that identified contact with animals, living on a farm or visiting farms to be important risk factors for STEC infections (85, 105, 112, 113). Differences in risk factors of STEC infections by age groups has also been reported (106, 114). For example, Werber et al. reported contact with a ruminant, playing in a sandbox and consumption of raw milk as risk factors for children under 3 years of age, while consumption of raw fermented spreadable sausages was a risk factor for individuals over 10 years (114). Friesema et al. identified contact with farm animals as a risk factor for non-O157 infections for cases under 10 years of age while consumption of beef was a risk factor for persons who were 10 years old and above in Netherlands (115).

When stratified by serotype, different risk factors for infection have also been observed, with international travel reported as a risk factor for non-O157 infections compared to O157 infections (116). This is in accordance with studies that have observed higher prevalence of non-O157 serotypes in geographical regions other than North America; indeed, in Europe, non-O157 serotypes are responsible for 80% diarrhea associated STEC cases (117). Another study identified consumption of hamburgers and occupational exposure to red meat associated with O157

infections while patients with non-O157 infections were more likely to have consumed sliced chicken and had occupational exposure to animals (112).

Non-Typhoidal *Salmonella* (NTS)

First discovered by Daniel E. Salmon and Theobald Smith in the 1800s, *Salmonella* are Gram-negative bacteria contributing to 1.2 million illnesses every year in the US (118). In 2015 alone, the incidence of *Salmonella* infections was estimated to be 15.74 per 100,000 population in the US (12).

The genus *Salmonella* is divided into two species: *S. enterica* and *S. bongori*, with approximately 2500 serotypes (119). *S. enterica* is divided further into six subspecies (120): *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) (Figure 1) and subspecies I (*enterica*) accounts for most of the clinical cases including the typhoidal and non-typhoidal serovars (119). The serovars Typhi, Sendai and Paratyphi belong to the typhoidal *Salmonella* group and are responsible for causing enteric fever, which is widely prevalent in the developing world (121). The remaining serovars are referred to as non-typhoidal *Salmonella* and are prevalent worldwide (121). The predominant NTS in the US include Typhimurium, Enteritidis, Newport and Heidelberg and in 2005 caused roughly 20.9%, 20.0%, 9.9% and 5.7% of infections, respectively (122, 123),. Globally, the serovars Enteritidis and Typhimurium are the most common causes of infection, contributing to 43.5% and 17.1% of the total number of *Salmonella* infections (122). In North America, Australia and New Zealand, Typhimurium was the most widely reported serovar, while in serovars Hadar and Agona were prevalent in European countries (122). The varying distributions and global epidemiology of *Salmonella* serovars highlights the need for continuous surveillance of these infections.

Pathogenesis and clinical presentation in humans

To cause disease in humans, food pathogens like *Salmonella* must utilize specific proteins that first allow it to overcome hostile environments such as the acidic stomach (124, 125), the anaerobic gastrointestinal tract (126) and the immune response mounted by the host (127). For instance, acid shock proteins (ASPs) such as RpoS and PhoP/PhoQ, the synthesis and/or uptake of compatible solutes during osmotic stress and the Fnr regulatory circuit for anaerobic metabolism are crucial for *Salmonella* pathogenesis (126). In addition, *Salmonella* pathogenicity islands (SPI), which are mobile genetic elements encoding various virulence factors, also play an important role in the pathogenesis of *Salmonella*. Overall 23 SPIs have been described so far, however, SPI-1 to SPI-5 are common in *S. enterica* serovars, each having an important function (127). SPI-1 and SPI-2 both encode a Type III Secretion System (T3SS), which are necessary for invasion of the intestinal epithelium, formation of a *Salmonella*-containing vacuole, and survival within macrophages (127). By contrast, SPI-4 encodes a Type I secretion system that helps the pathogen adhere to epithelial cells (127).

Once in the intestines, *Salmonella* crosses the epithelial cell barrier either by invading the cells using effector proteins secreted by the SPI-1 T3SS (127). The SPI-1 T3SS encodes effector proteins such as SipA, SipC, SopB and SopE2 which result in actin cytoskeleton remodelling resulting in membrane ruffling of host cells and thus internalization into host cells (128). In addition, *Salmonella* can also cross the epithelial cell barrier via passive transport using dendritic cells; the migration of *Salmonella* infected dendritic cells to mesenteric lymph nodes facilitates the spread of *Salmonella* to different organs (129). The membranous epithelial (M) cells are an important target site for invasion by *Salmonella*, which can get translocated across the intestinal epithelium and into the underlying follicles and mesenteric lymph nodes (127, 130). After crossing

the intestinal epithelial barrier, the bacteria are engulfed by macrophages forming a *Salmonella*-containing vacuole (SCV) (131). At this stage, effector proteins such as SigD/SopB, SipA, SipC, encoded by SPI-2, are secreted into the cytosol of macrophages to help prevent the fusion of the SCV with the lysosome (127). After macrophages containing bacteria undergo apoptosis, *Salmonella* can re-invade epithelial cells or be engulfed by other phagocytic cells (127).

In humans, *Salmonella* infection can result in fever, gastroenteritis, bacteremia with or without additional complications and can lead to a chronic carrier state. While the typhoidal serovars of *Salmonella* are responsible for enteric fever, NTS cause gastroenteritis and invasive NTS infections (119). The ingestion of more than 10^5 *Salmonella* cells (132) is required to cause the disease, however studies have reported doses less than 10^3 cells of *Salmonella* responsible for causing outbreaks (133). Although many serovars are responsible for causing gastroenteritis, *S.* Typhimurium and *S.* Enteritidis are the leading causes of gastroenteritis in the US (119). Approximately 6-72 hours after ingesting the organism (134), patients may experience fever, diarrhea, and abdominal cramping (135). While gastroenteritis is usually a self-limiting condition and resolves quickly upon treatment with fluids and electrolyte therapy, about 5-8% of gastroenteritis cases will develop into bacteremia, or the presence of bacteria in the bloodstream (119, 135). This invasive extra-intestinal infection is often associated with immunocompromised patients and can result in complications such as hepatomegaly (enlargement of the liver) and splenomegaly (enlargement of the spleen) (121). Invasive NTS infections are more likely to be caused by serovars Typhimurium, Dublin, and Choleraesuis than other serovars (119, 121), though there is considerable variation in the geographic distribution of invasive infections with the highest number of invasive NTS infections occurring in Africa followed by Europe (136). Chronic carriage

is not common for the NTS serovars as only 0.1% of NTS isolates have been detected in stools for periods of more than a year (119, 121).

Reservoirs of NTS and its transmission to humans

Different serovars of *Salmonella* are associated with different host populations; these serovars may be host adapted or have a ubiquitous host range. Serovars such as Typhi and Paratyphi are exclusively adapted to humans, while others such as Gallinarum and Abortusovis are adapted to poultry and sheep, respectively (132). These serovars are host-restricted since they are exclusively associated with one host species (137). Furthermore, serovars such as Typhimurium and Enteritidis have a broad host range and the ability to adapt to multiple unrelated host species including humans, poultry and wild rodents (132). Numerous studies have documented the presence of NTS *Salmonella* in poultry products, farms and processing environments (138-140). For example, Singh et al. reported *S*. Typhimurium to be the predominant serovar in chicken eggs in North India (141), while a study in Malaysia documented a high prevalence of various *Salmonella* serovars from multiple sources including chicken carcasses, defeathering machines and transport cages. The serovars Albany, Corvallis and Brancaster were predominated among the isolates identified in Malaysia (142). Additionally, wild birds are natural reservoirs of NTS; *S*. Enteritidis was isolated from wild waterfowls in Ukraine (143). Cattle (123, 144, 145) and pigs (146-148) have also been reported to be important reservoirs for serovars such as Typhimurium, Enteritidis and Derby.

NTS infections are mostly transmitted to humans via contact with specific animals or via the consumption of contaminated food and water. Since animals play an important role in the infection cycle of *Salmonella* by serving as reservoirs, it is likely that contact between colonized

animals and humans is a major source of human NTS infections (149). Indeed, Fone et al. identified associations between diseases in farming families and contact with animal infections such as cattle and sheep; the authors identified nine of the 23 human infections with *S.* Typhimurium phage definitive type DT104 to have had contact with animals or individuals working closely with animals (150). Similarly, Hendriksen et al. isolated *S*. Typhimurium strains from a pig, calf, and child that were phenotypically and genotypically identical, thus alluding to transmission via animal contact (151). Contact with domestic animals have also been suggested to be an important source of NTS infections in humans (152).

Although animal contact is an important source of NTS infections, consumption of contaminated food products is also a major source of NTS infections. Indeed, the CDC has reported many outbreaks of *Salmonella* associated with food products such as raw turkey products, raw sprouts, chicken salad and frozen shredded coconut (153). In recent years, an increase in *Salmonella* outbreaks due to contaminated fruits and vegetables has been observed in the US (154). Produce may be contaminated by *Salmonella* either due to cultivation practices and at the processing stages (155) or due to the use of *Salmonella* contaminated manure and irrigation water (101). In addition, *Salmonella* has also been isolated from water, thus making waterborne transmission an important route for human infections (156). Interestingly, Gast et al. also documented airborne transmission of *Salmonella* between groups of chicks (157), which has concerning implications on human health. The identification of shared genotypes and antimicrobial susceptibility phenotypes among NTS isolates recovered from both the environment and humans provides support for the environment as an important source of NTS infections in humans (158).

Risk factors of NTS infections

Although NTS belong to the same genus, there is considerable variability amongst serovars in terms of characteristics such as host adaptability and virulence. To better manage diseases caused by different serovars, many studies have identified serovar-specific risk factors. Since animals serve as reservoirs of NTS infections, animal contact has been reported to be an important risk factor for NTS infections (159-162). While looking at *Salmonella* infections in New York and Washington, Cummings *et al*. identified farm animal contact to be significantly associated with salmonellosis; specifically, contact with cattle five days prior to infection was a risk factor (163). In addition, a study looking at NTS infections in Michigan identified contact with reptiles and cats to be significantly associated with infections in children (164). Mughini-Gras et al. identified serovar-specific risk factors of salmonellosis in Netherlands as consumption of raw/undercooked meat and prior antibiotic use were risk factors for pig-associated salmonellosis and consuming raw/undercooked eggs was a risk factor for poultry-associated salmonellosis (160). Similarly, Doorduyn et al. identified serovar-specific risk factors for Typhimurium and Enteritidis infections, with the use of proton pump inhibitors and consumption of raw eggs or products containing raw eggs to be associated with Enteritidis infections. Use of antibiotics, playing in a sandbox, occupational exposure to raw meat, and consumption of undercooked meat, however, were associated with Typhimurium infections (165). Consumption of peanut butter or products containing peanut butter (166) as well as consumption of sprouts (167) have been linked to NTS infections. Considering the importance of invasive NTS infections, several studies have also identified risk factors for invasive NTS to help in disease management. Thamlikitkul et al., for instance, identified acquired immunodeficiency syndrome (AIDS) and corticosteroid use as risk factors of NTS bacteraemia in Thailand (168). HIV infection, malnutrition, malaria, young age,

anaemia and residence in a rural setting have also been reported as risk factors for invasive NTS infections in Africa (169).

ANTIBIOTIC THERAPY FOR TREATMENT OF ENTERIC INFECTIONS

Although most enteric infections are self-limiting and require fluids and electrolyte therapy, antimicrobial agents play an important role in treatment of enteric infections. Antibiotics such as macrolides, fluoroquinolones, trimethoprim-sulfamethoxazole and third generation cephalosporins have been employed against important enteric pathogens such as *Campylobacter*, *Shigella*, non-typhoidal *Salmonella* and non-Shiga toxin-producing *E. coli* (170).

Antibiotics are not recommended for treatment of STEC infections as their role in the development of severe disease outcomes is debatable (171-173). The treatment of STEC infections with antibiotics has been shown to result in increased toxin production, thus increasing the risk of development of haemolytic uremic syndrome (HUS) (174, 175). The treatment of *E. coli* O157:H7 infected mice with fluoroquinolone antibiotics, for instance, detected high levels of toxin in feces and was also associated with higher frequencies of death compared to mice treated with fosfomycin (174). Fluoroquinolones and other antibiotics that inhibit DNA replication trigger the SOS response in bacteria, thus inducing the Stx prophage and ultimately toxin production (174, 176, 177), though this response varies depending on the type of antibiotic used. Antibiotics that target the cell wall, transcription, or translation were not shown to induce toxin production, indicating that specific antibiotics may not be contraindicated for patients infected with STEC infections as significantly lower levels of toxin were produced than antibiotics targeting DNA synthesis (178). In addition, an age-matched case-case comparison study in Minnesota found no association between antibiotic treatment and development of HUS (179), which is specifically linked to toxin

production. Despite the CDC recommendation to avoid antibiotics for the treatment of STEC, some studies have documented the use of antimicrobial and antimotility agents for STEC O157 infections (180). Nelson et al. reported that 62% of 474 patients, living in the FoodNet surveillance sites in the US, infected with STEC O157 were treated with an antimicrobial agent from 1996-1997 and in 1999; fluoroquinolones, trimethoprim-sulfamethoxazole and β-lactam antibiotics were observed to be commonly prescribed to patients with STEC O157 (180).

For the treatment of salmonellosis, antimicrobials such as fluoroquinolones, third generation cephalosporins, penicillins, macrolides and trimethoprim- sulfamethoxazole are commonly prescribed, particularly in the immuno-compromised, the young and the elderly (181, 182). Although fluoroquinolones are effective drugs against NTS, the FDA does not recommend use in children due to studies observing arthropathy, or joint disorders, in juvenile laboratory animals (181, 183-186). Thus, alternative antibiotics such azithromycin and trimethoprim-sulfamethoxazole are administered to children for treatment of NTS (170). Numerous studies have been conducted that have conflicting views on the benefits of antimicrobial therapy for treating NTS infections. When compared to a placebo group, treatment with ciprofloxacin significantly reduced the duration of diarrhea and other symptoms in patients (181). Furthermore, antimicrobial therapy with ciprofloxacin has also been shown to be effective in controlling *Salmonella* outbreaks (187). However, other studies have documented no real benefit of antimicrobial therapy for treating *Salmonella* infections (188, 189). Although Sirinavin et al. found that antibiotic therapy resulted in more negative cultures, they did not find any significant differences in duration of illness and diarrhea between placebo and antibiotic therapy (190). Thus, antimicrobial therapy for treatment of salmonellosis is not routinely recommended to all patients and is only administered

to patients with risk factors such as HIV infection, those undergoing therapeutic immunosuppression, the young, elderly, and those at risk for extra intestinal infections (181).

ANTIBIOTIC RESISTANCE IN ENTERIC PATHOGENS

The CDC estimates that approximately two million resistant infections occur annually in the US, resulting in 23,000 deaths (191). Resistant infections contribute to twice as much of the morbidity and mortality as well as the cost of hospitalizations (192, 193). Unfortunately, by 2050**,** 10 million deaths due to antimicrobial resistant infections is projected to occur annually, which may drive antimicrobial resistance to be the leading cause of death worldwide (194). It is therefore imperative to identify novel drugs capable of treating resistant infections and continue efforts for the judicious use of antibiotics in order to optimize drug effectiveness.

To detect and monitor resistant infections, antimicrobial susceptibility testing has been standardized using the guidelines established by the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). CLSI defines "resistant" *Enterobacteriaceae* as strains with a minimal inhibitory concentration (MIC) of ampicillin as $\geq$32 µg/mL, while the MIC of ciprofloxacin is $\geq$1 µg/mL for *Salmonella* and $\geq$4 µg/mL for all other *Enterobacteriaceae*. The MIC for trimethoprim-sulfamethoxazole is defined as $\geq$4/76 µg/mL, $\geq$16 µg/mL for tetracycline, and $\geq$4 µg/mL for third generation cephalosporins such as ceftriaxone (195).

The emergence and spread of antimicrobial resistance is intricately linked to human, animal and environmental factors. Indeed, resistant infections can spread in the community via direct or indirect contact with people harbouring resistant infections, contact with animals that serve as reservoirs of resistant bacteria, and the consumption of food products contaminated with resistant

organisms (Figure 2). The surveillance of antibiotic resistant infections is crucial to detect and control the worldwide spread of antibiotic resistant bacteria. In the US, the National Antimicrobial Resistance Monitoring System (NARMS) provides data on the trends and frequencies of antibiotic resistant infections in important food pathogens. NARMS was established in 1996 as a collaborative effort between the state and local public health departments in the US, the CDC, the FDA and the USDA. The NARMS surveillance system tracks antibiotic resistance frequencies and trends in *Salmonella*, *Campylobacter*, *Shigella*, *E. coli* O157, and *Vibrio* species isolated from patients, food sources and food animals (196). The 2011 NARMS Executive Report (197) highlights important changes in the trends of antibiotic resistance in NTS, *E. coli* and *Campylobacter*. Importantly, frequencies of resistant NTS in 2011 were lower than the average of the years 2003-2007. Additionally, frequencies of ciprofloxacin resistant NTS isolated from humans were less than 0.5%. Multidrug resistant NTS from humans were also found to have decreased in 2011 (9.1%) when compared to the average frequencies from 2003-2007 (12.1%); serovars I 4, [5],12: i:- and Heidelberg were found to have high frequencies of multidrug resistance. In addition, high frequencies of resistance to ceftriaxone in *E. coli* isolates from food sources was documented in this report. While the 2011 NARMS Executive Report does not report antibiotic resistance frequencies from *E. coli* such as STEC, isolated from humans, the NARMS Now interactive tool (198) contains antibiotic resistant data for enteric pathogens isolated from humans.

In Europe, the European Centre for Disease Prevention and Control manages the European Antimicrobial Resistance Surveillance Network (EARS-Net), which collects antimicrobial resistance data for select pathogens from 30 participating countries and analyses spatial & temporal trends in antimicrobial resistance (199). In 2016, EARS-Net reported 58.6% *E. coli* isolates to be resistant to at least one antibiotic; no data was available for NTS since it is not included in this

surveillance system (200). In 2015, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) to promote global surveillance and strengthen research on antimicrobial resistance using genotypic, phenotypic, epidemiological data, clinical data and population-based data (201). As mentioned in the GLASS Early Implementation Report, surveillance of antimicrobial resistance and assessing factors associated with resistance is crucial to develop policies and interventions to manage the spread of antibiotic resistant infections worldwide (202).

Numerous studies have documented antimicrobial resistance in both STEC and NTS worldwide. In 1994, Kim et al. found 7.4% of STEC O157:H7 isolates from Washington to be resistant to tetracycline, streptomycin and sulfisoxazole. Since these antibiotics are not widely used in human medicine, it was suggested that antibiotic resistance in these isolates was unlikely to have originated in humans (203). However, recent studies have shown an increase in resistance among STEC O157 isolates to clinically important antibiotics such as ampicillin, trimethoprim-sulfamethoxazole and third generation cephalosporins (204-209), suggesting the widespread and empirical use of antibiotics for treatment of enteric infections (210, 211), including STEC, may be contributing to the emergence of antibiotic resistance in STEC. Schroeder et al. noted high frequencies of resistance to antibiotics important in human and veterinary medicine in swine-derived STEC O157 isolates compared to human isolates (205), while Meng et al. documented high frequencies of antibiotic resistance to clinically and agriculturally relevant antibiotics from cattle-derived STEC isolates (34%) (206). Considering the widespread use of antibiotics in agricultural settings, these studies suggest that animals play an important reservoir for antimicrobial drug resistant STEC infections. Non-O157 STEC isolates recovered from both humans and animals have also been found to be resistant to antibiotics such as ampicillin,

tetracycline and trimethoprim-sulfamethoxazole commonly used in human and veterinary medicine (212, 213). Interestingly, studies have found higher frequencies of antibiotic resistance in non-O157 serotypes of STEC compared to STEC O157 (214, 215). The reason behind this difference in resistance frequencies between STEC serotypes is unclear. Both O157 and non-O157 serotypes have been reported to be prevalent and able to persist in the environment (216, 217) and are likely to take up antibiotic resistance genes present in the environment (218, 219). Genomic plasticity in different NTS serovars has been offered as a potential explanation for differences in antibiotic resistance frequencies observed between NTS serovars (220); further studies need to be conducted to test this hypothesis for STEC isolates.

With 100,000 drug resistant NTS infections occurring every year in the US and resulting in $365,000,000 in medical costs, the CDC considers drug-resistant NTS infections to be a serious global threat requiring prompt attention (221). Medalla et al., for instance, calculated the incidence of ampicillin resistant infections to be 1.07/100,000 person-years, 0.51/100,000 person-years for ceftriaxone and ampicillin resistant infections, and 0.35/100,000 person-years for ciprofloxacin resistant infections in the US (222). A study in Spain also documented high frequencies of resistance in *Salmonella* Typhimurium isolates to ampicillin (76%), sulphonamides (78.7%) and tetracyclines (80.4%) (223), while a significant increase in trimethoprim-sulfamethoxazole and nalidixic acid resistance was detected in NTS isolates over a 15-year period in Thailand (224). Antibiotic resistance to third generation cephalosporins has also been reported worldwide (225, 226), which is concerning considering its importance in treatment of NTS infections in children and the elderly. Antibiotic resistant NTS have been isolated from food animals such as poultry (227), swine, and cattle (228, 229), highlighting the importance of food and the farm environment as a source of resistant infections for humans. Importantly, the emergence of multidrug resistant

Salmonella has been documented and attributed to the extensive use of antibiotics in clinical and agricultural settings (230, 231). A Danish study looking at NTS isolates from pigs observed ampicillin-streptomycin-sulphonamide-tetracycline resistance as the predominant multidrug resistance profile (230). The MDR pattern ampicillin, chloramphenicol, streptomycin, sulfisoxazole and tetracycline (ACSSuT) is widely prevalent in *S.* Typhimurium DT104 and has also been associated with resistance to β-lactam drugs such as ceftriazone and amoxicillin-clavulanic acid (197). The emergence of multidrug resistance in NTS isolates is alarming as it limits the repertoire of antibiotics that can be used for antimicrobial therapy. Antibiotic resistant NTS have been found to be associated with severe disease outcomes. One such study in western Kenya found multidrug resistant NTS infections to be significantly associated with bacteremia compared to diarrhea (232). MDR NTS have been reported to be associated with more serious outcomes such as increased bloodstream infections, more hospitalizations and higher mortality compared to antibiotic sensitive strains (233). In addition, a study in the US, observed that NTS isolates resistant $\geq$1 antibiotic were significantly more likely to cause bloodstream infections compared to isolates that were susceptible (234). In addition, hospitalization due to bloodstream infections was also identified to be significantly associated with antibiotic resistant NTS (234). Furthermore, a study looking at NTS isolates from Oregon also identified hospitalization more likely in patients with resistant infections than those with susceptible infections, and the authors also identified that patients with resistant NTS infections were more likely to have travelled to eastern or Southeast Asia (235)

RISK FACTORS ASSOCIATED WITH ANTIBIOTIC RESISTANT STEC AND NTS INFECTIONS

Many epidemiological studies looking at risk factors of antibiotic resistant infections have been conducted worldwide. Although antibiotic resistant enteric pathogens are a global health problem requiring prompt attention, there is a dearth in studies that identify risk factors of antibiotic resistant enteric infections.

Previous antibiotic exposure has been found to be an important risk factor of antibiotic resistant infections in many independent studies (236-242). For example, Hillier et al. studied risk factors of *E. coli* causing urinary tract infections (UTI) and observed that ampicillin resistant and trimethoprim resistant *E. coli* infections were associated with prior use of amoxicillin and trimethoprim for ≥7 days in the past month (237). In addition, a study in the US identified the relative risk of infection with an extended spectrum β lactamase (ESBL), third generation cephalosporin, or trimethoprim-sulfamethoxazole resistant *E. coli* or *K. pneumoniae* isolate to be higher in patients with antibiotic use up to a month prior to the infection compared to those with no antibiotic use (236). In another study of community cases of Gram negative UTIs in Scotland, Steinke et al. determined that prior trimethoprim use was more common in cases positive for trimethoprim-resistant bacteria (241). A previous study has identified prior antibiotic use to be a risk factor for antibiotic resistant NTS infections; patients with antibiotic resistant NTS infections were 5 times more likely to have taken the respective antibiotic a month prior to illness (243). Seidman et al., however, did not find prior medication use to be a risk factor for antibiotic resistance in faecal *E. coli* isolated from children in rural India (244). Similar observations were noted by Fosnani et al. as they did not observe any association between antimicrobial use and trimethoprim-resistant faecal *E. coli* in children and their household members (245). Thus,

additional studies are warranted to determine whether prior antibiotic use is a risk factor for antimicrobial resistant STEC and NTS infections.

Several studies have identified demographic characteristics to be associated with antibiotic resistant infections. One study, for instance, identified significantly higher frequencies of methicillin resistant *Staphylococcus aureus* (MRSA) infections in African American patients than in white or Hispanic patients (246), while resistance frequencies in *Pseudomonas aeruginosa* differed by age group and the antimicrobial agent. Specifically, resistance frequencies were significantly higher in patients between 18 and 39 years of age relative to patients $\geq$ 70 years of age (247). For UTI causing *E. coli*, a prospective study in Spain identified patients over 50 years of age to have significantly higher frequencies of nalidixic acid and fluoroquinolone resistant *E. coli* infections than patients aged <50 years of age (248). The same study identified males to have significantly higher frequencies of fluoroquinolone resistant infections than females (248). Not many studies have identified risk factors of antibiotic resistant enteric infections, especially resistant STEC infections, thus it is unknown whether demographic variables are linked to these resistant infections. However, age has been reported to be associated with antibiotic resistance in NTS with significantly higher frequencies of resistance to multiple antibiotics in children < 5 years of age (249).

Other epidemiological factors have also been shown to be associated with antibiotic resistant infections. For example, Shorr et al. found resistant pneumococcal infections to be independently associated with recent hospitalization, residence in a nursing home, long-term hemodialysis and ICU admission (250). In addition, a prospective study in France identified prior use of a urinary catheter in the last one year to be a significant risk factor for antibiotic resistant *E. coli* infections (242). As mentioned earlier, only a few studies have been conducted to identify risk

factors of antibiotic resistant enteric infections. For example, Cha et al. identified foreign travel to be associated with fluoroquinolone resistant *Campylobacter jejuni* infections (251). Fluoroquinolone resistant *Campylobacter* infections were also found to be associated with the consumption of poultry products prepared at commercial establishments (252). For antibiotic resistant NTS infections, geographical variables have also been found to be associated with resistant NTS infections; Odoch et al. reported significant differences in frequencies of antibiotic resistance in NTS from hens in various districts in Uganda (159). Additional studies have noted the geographical variation in antibiotic resistance. For example, Sahoo et al. identified higher odds of antibiotic resistance in *E. coli* from fecal samples, cow dung and drinking water samples from non-coastal areas in India when compared to coastal areas, which they attributed to social and environmental variables (253). Geographical variability in antibiotic resistance can be explained by many factors such as antibiotic usage in different countries (254), public health factors such as antibiotic usage policies (255, 256) and by the spread and distribution of antibiotic resistant pathogens in different geographic regions (257). Due to the differences in such factors in geographic regions, risk factors of resistant infections may vary, thus highlighting the importance of continued surveillance of risk factors of antibiotic resistant infections.

While few studies have looked at risk factors for antibiotic resistant NTS infections, a review of the literature revealed no reports on risk factors associated with antibiotic resistant STEC infections. Considering the emergence of antibiotic resistance in STEC and the importance of antibiotic resistant NTS infections, it is imperative that risk factors of these antibiotic resistant infections are identified for the development of novel intervention strategies.

THE MOLECULAR EPIDEMIOLOGY OF ANTIBIOTIC RESISTANT INFECTIONS

Molecular epidemiology has been applied by many scientists to study the occurrence of antibiotic resistant genes (ARGs), and to understand the dynamics and transmission of antibiotic resistant infections in important bacterial pathogens (258-261). Due to the increasing threat of antibiotic resistant infections, scientists have utilized molecular epidemiological tools to study the global spread of antibiotic resistance in an effort to enhance the management of resistant infections (262-264).

Differences in antibiotic resistance frequencies by bacterial serotypes and serovars has been reported. For example, NTS serovars were found to be associated with antibiotic resistance, with many studies observing significantly lower frequencies of resistance in Enteritidis serovars compared to other serovars (223, 235, 265). In addition, Perron et al. observed associations between antimicrobial drugs and serovar in *S. enterica* isolates from swine, with resistance to ampicillin, chloramphenicol, tetracycline and trimethoprim-sulfamethoxazole significantly associated with Typhimurium, while resistance to cefoxitin, cefalotin and ceftiotur was found to be associated with Heidelberg serovars (266). Similarly, differences in antibiotic resistance frequencies have also been observed in STEC, with the O157 STEC serotypes having lower levels of resistance than the non-O157 serotypes (214, 215). While the reasons for these differences in resistance frequencies is unclear, differing resistance mechanisms and understanding the molecular epidemiology of resistant infections may provide answers to these questions.

The acquisition and dissemination of horizontally transmitted ARGs are of concern as they can spread to other clinically relevant pathogens and commensal organisms. Numerous studies have been performed in both STEC and NTS to determine the prevalence and diversity of ARGs.

Earlier studies in the 1980s have not observed STEC isolates to be resistant to many antibiotics; Ratnam et al. reported 97% of STEC O157:H7 isolates from Canada and US to be susceptible to commonly used antibiotics, while Bopp et al. did not observe any *E. coli* O157:H7 strain to be resistant to any of the 12 antibiotics tested (267, 268). However, ARGs have likely played an important role in the emergence and spread of resistance in STEC (269). Since, antibiotics are not prescribed for the treatment of STEC infections, it is also important to note that the lack of surveillance for antibiotic resistance in STEC may have resulted in underestimating resistance frequencies. In 2011, a large outbreak of STEC O104:H4 causing many HUS cases occurred in Germany (270); further genomic analysis of this strain determined the presence of a plasmid encoding a CTX-M-15 β-lactamase conferring resistance to penicillins and cephalosporins (271). The CTX-M extended spectrum β-lactamase (ESBL) has also been reported in many other serotypes of STEC (272, 273). In addition, multi-drug resistant STEC isolates carrying multiple antibiotic resistance genes have also been reported. For example, while looking at STEC isolates from humans, animals and food products, Zhao et al. observed the presence of integrons containing *aadA* and *dfrXII* conferring resistance to streptomycin-spectinomycin and trimethoprim respectively (274). Multi-drug resistant clinical STEC O118 strains from Europe carrying at least one of the following genes: *aphA1*-Ia, *catA1*, *tet(A)*, and *blaTEM-1*, *sulI*, *aadA1a*, or *dfrA1* conferring resistance to kanamycin, chloramphenicol, tetracycline, ampicillin, sulphonamide, streptomycin and trimethoprim respectively, has also been reported (275). For *Salmonella*, one study of isolates from humans, poultry and seafood to possess different ARG profiles, with few strains containing more than 10 resistance genes conferring resistance to different antibiotics (276). Another study of *S*. Typhimurium DT104 isolates from Denmark were found to carry two separate integrons encoding different resistance genes such as *sul1*, *ant (3'')-Ia* and β-lactamase gene

encoding resistance to sulphonamides, streptomycin & spectinomycin and penicillins, respectively (277). In addition to horizontally transmitted genetic elements, mutations in chromosomal genes such as *glpT* and *gyrA* conferring antibiotic resistance to fosfomycin and fluoroquinolones have also been reported in both STEC and NTS (278-280).

Molecular typing is an important tool to study the evolution and phylogenetic relationship among bacteria. These tools have a variety of applications such as in surveillance of infectious diseases, in outbreak investigations and identifying sources of transmission of infections. Additionally, many studies have documented the use of molecular typing and genomic analysis in tracking the spread of antibiotic resistance and identifying genetic determinants of resistant infections (251, 278, 281-284). Tools used commonly for genetic characterization of bacteria include Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST), and Single Nucleotide Polymorphisms (SNP) typing. PFGE is the 'gold standard' method for genotypic characterization of pathogens such as *E. coli*, *Campylobacter* and *Salmonella* (285); however, it has several disadvantages such as the subjective analysis of banding patterns and being extremely labour intensive. This makes MLST a more attractive alternative which uses the sequences of seven or more house-keeping genes to characterize bacteria and separate them into sequence types (STs) (286). MLST has been used to determine the genetic diversity of non-O157 STEC isolates and NTS. Several such studies revealed the presence of many different STs of cattle non-O157 STEC isolates (287) and the occurrence of certain non-O157 STs in both humans and animals (288). *Salmonella* subtyping using MLST has been used to study the genetic diversity of bovine and human isolates in Michigan (289), shedding light on the temporal changes in the distribution and circulation of *Salmonella* subtypes in the environment. However, since MLST was unable to determine the genetic diversity for STEC O157 isolates (290), a SNP typing tool

was developed by Manning et al. which detected variation in up to 96 SNP loci for *E. coli* O157 and revealed the association of clades with disease severity (291).

Due to the importance of determining the prevalence of antibiotic resistant phylogenetic lineages to help in disease management strategies, many studies have been published exploring the association between phylogenetic lineages of different genera of bacteria and antibiotic resistance. Using a combination of the genetic markers *chuA*, *yjaA* and DNA fragment TspE4.C2, *E. coli* strains have been assigned to four phylogenetic groups: A, B1, B2 and D (292, 293). While this phylogenetic classification system has been employed to characterize *E. coli* strains, track their spread and identify factors associated with them (294-296), studies have also looked at the relationship between phylogenetic lineages of *E. coli* and resistance to different antibiotics. For example, Moreno et al. determined that clinical isolates of uropathogenic *E. coli* from Spain belonging to phylogenetic group A were significantly associated with quinolone and fluoroquinolone resistance, while those belonging to phylogenetic group D were associated with resistance to trimethoprim-sulfamethoxazole (297). Moreover, on examining *E. coli* from human clinical samples in Iowa, Johnson et al. noted that isolates belonging to phylogenetic groups A and D were significantly associated with fluoroquinolone resistance (298). Similarly, other studies looking at the associations between antibiotic resistance and phylogenetic groups of *E. coli* from different sources have been conducted (299, 300). While looking at the phylogenetic background, virulence gene profiles and antibiotic resistance of non-H7 enteropathogenic *E. coli* O157, Ferdous et al. observed that *E. coli* O157 strain Santai, which belongs to phylogenetic group D, was resistant to multiple drugs (301). In addition, *E. coli* sequence type (ST)-131, which belongs to phylogenetic group B2, has been observed to be associated with resistance to fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole (302, 303). Among diarrheagenic *E. coli*

(DEC) strains from Peru, which included enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC), Mosquito et al. reported significant differences in frequencies of resistance to trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, nalidixic acid and multidrug resistance among different phylogenetic groups. Specifically, higher frequencies of resistance observed in phylogenetic groups B2 and D (304). Although no association between phylogenetic lineages and genetic determinants of resistance in atypical enteropathogenic *E. coli* (aEPEC) strains from South Asia and sub-Saharan Africa was observed, the authors did find an association between geographical location and genetic determinants; for example, *dfrA1* genes were most common in Asia and *dfrA14* and *dhfr7* were more common in Kenya, while the *tet(A)* gene was prevalent in West and East Africa compared to Asia (305) .

While studies have been conducted looking at factors, such as virulence genes and severe disease outcomes, associated with phylogenetic lineages in STEC (291, 306), not many studies have looked at associations between antibiotic resistance and phylogenetic lineages in STEC. Using phage typing to classify STEC O157:H7 isolates, Mora et al., for instance, observed certain STEC O157:H7 phage types (PT) to be linked with higher frequencies of antibiotic resistance; 54% of PT2 isolates and 75% of PT23 isolates were resistant to antibiotics tested in the study (215). Similarly, few studies have looked at the association between antibiotic resistant infections and phylogenetic lineages of NTS. For example, a novel typing tool, based on two virulence genes, *fimH* and *sseL*, and clustered regularly interspersed short palindromic repeats (CRISPRs) known as CRISPR-MVLST (multi-virulence-locus sequence typing) (307), was used to type *S.* Typhimurum isolates into STs; *S.* Typhiumirum isolates were classified into 22 STs and some sequence types were found to be associated with resistance to antibiotics (308). In addition,

Alcaine et al. reported distinct evolutionary lineages of *S. enterica* to be more likely to possess the plasmid *bla*-$_{\text{CMY-2}}$ conferring resistance to ceftriaxone and ceftiofur. Interestingly, only one of the two *S. enterica* serovar Agona MLST lineages comprised all ceftiofur resistant isolates containing the *bla*$_{\text{CMY-2}}$ plasmid (309). Other studies have also reported the spread of *S. enterica* genotypes conferring resistance to important antibiotics (310, 311). For instance, Izumiya et al. reported that 39.8% of the total 221 multidrug resistant *S.* Typhimurium serovars, with multiple resistant patterns, belonged to definitive phage type DT104 (310). Vatopoulos et al. observed 91.3% of a total of 23 ampicillin resistant *S. enteritidis* strains in Greece belonging to phage type 6a (311).

Studies identifying links between phylogenetic lineage and antibiotic resistance have also been reported in other pathogens. Indeed, a recent study on antibiotic resistant phylogenetic lineages of *C. jejuni* found that, when compared to other phylogenetic lineages, strains belonging to multilocus sequence type (ST)-464 were significantly associated with ciprofloxacin and nalidixic acid resistance, while ST-982 strains were more likely to be resistant to tetracycline (251). In addition, a study looking at *Mycobacterium tuberculosis* isolates from the US identified the East Asian lineage more likely to be resistant to fluoroquinolones when compared to other lineages (312). Another study looking at association between *M. tuberculosis* lineages and antibiotic resistance in Switzerland found the East Asian Lineage 2 (which includes Beijing lineage) to be significantly associated with resistance to any drug (257).

ANTIMICROBIAL USE IN ANIMALS AND IMPLICATIONS ON HUMAN HEALTH

In food animals, antibiotics are used routinely for therapeutic purposes, disease prophylaxis, disease prevention, for growth promotion and feed efficiency (313, 314). The FDA estimates that in 2016, approximately 13.98 million kilograms (kgs) of antimicrobials were sold

and distributed in the US for use in food producing animals (315). There are currently 19 antimicrobial classes that are approved for use in food producing animals out of which six are also widely used in non-food producing animals (315). Importantly, approximately 8 million kgs of the total 13.98 million kgs of antimicrobials sold in the US are commonly used in human medicine; 3.5 million kgs of these medically important antimicrobials were used in cattle alone (315). Among the medically important antibiotics, 70% of the total sales were for tetracycline and 10% were for penicillin (315). High antimicrobial use in animals has also been documented in other countries. The Canadian Antimicrobial Resistance Surveillance System (CARSS), for instance, reported use of 1 million kilograms of medically important antimicrobials in food producing and non-food producing animals in 2016 (316). Additionally, in 2010, the global use of antimicrobials in food animal production was estimated to be 63,151 tons (57 million kgs) with China, US, Brazil, India and Germany documented as the largest consumers of antimicrobials in food production. Alarmingly, the use of antimicrobials has been projected to increase to 105,596 tons (95 million kgs) by the year 2030 (317).

This high rate of antimicrobial use is an important contributor to the emergence and spread of antimicrobial resistance in different environments (221). It has been shown that antibiotic residues can remain in the environment and contribute to the persistence of antibiotic resistant bacterial populations (318). In addition, agricultural antibiotic use was identified to be instrumental in the emergence of antibiotic resistance in commensal bacteria (319). The use of antibiotics has also been shown to have a profound effect on the microbiome and mobilome of animals; the diversity and abundance of antimicrobial genes increased in the microbiome of swine given antibiotics when compared to swine not given antibiotics in their diet (320).

Numerous studies have been conducted to further understand the consequences of antimicrobial use in food animals. One of the earliest studies observing a correlation between antibiotic use in animals and the emergence of antibiotic resistance was conducted in 1951; streptomycin resistant coliforms were isolated from the guts of turkeys fed streptomycin as a growth promoting antimicrobial (321). Similarly, Chantziaras et al. identified high correlations between the use of sulphonamides, fluoroquinolones and penicillins and resistance to corresponding antibiotics in *E. coli* in seven European countries; Belgium had the highest veterinary use of antibiotics and was also reported to have the highest levels of resistance (322). Banning antibiotics such as virginiamycin in food animals was shown to reduce the frequencies of antimicrobial resistance to erythromycin and virginiamycin in *Enterococcus* in Denmark (323). Use of subtherapeutic levels of antibiotics in farm animals has also been suggested to contribute to the persistence of antibiotic resistant bacteria in soil. Ghosh et al., for instance, observed significantly higher frequencies of chlortetracycline resistance among soil bacteria in a farm where manure from antibiotic-treated animals was allowed to accumulate (324). Interestingly, Endtz et al. noted that the introduction of the fluoroquinolone, enrofloxacin, for use in poultry coincided with the emergence of fluoroquinolone resistant *C. jejuni* infections in Netherlands (325).

An important consequence of antimicrobial use in animals is the spread of resistant infections to humans. Indeed, numerous studies have observed the association between antibiotic use in food animals and antibiotic resistant bacteria in humans. Fein et al. reported identical antibiogram patterns of *E. coli* isolated from people and animals living on the same farm (326). Additionally, vancomycin resistant isolates from clinical and non-human sources were found to share ribotype patterns, suggesting that there may be transmission of resistant infections between animals and humans (327). Reynaga et al. observed similar *spa* types and antibiograms in

methicillin resistant *Staphylococcus aureus* (MRSA) in pigs and farmers and also suggested the transmission of antibiotic resistant strains between animals and humans, though the direction of transmission was not examined (328). Additionally, the spread of antibiotic resistant bacteria to humans via contaminated food products is of great concern (329-332). Comparing between organic and conventionally-raised chickens in Maryland, for example, Cui et al. observed higher frequencies of resistance in *S. enterica* serovar Typhimurium isolates from the conventional chickens; most isolates from organic chickens were susceptible to the 17 antibiotics tested (333).

Since animals are an important reservoir of STEC and antibiotics are widely used in agricultural settings, it is likely that animals serve as an important reservoir of antibiotic resistant STEC. One study of STEC O157 and non-O157 STEC isolated from domestic animals in rural communities in North-western Mexico, for instance, documented resistance to ampicillin, cephalothin, chloramphenicol and kanamycin (334). STEC isolates from a variety of different sources including dairy cow feces, bovine feedlot cows and bovine dairy have also been found to be resistant to multiple antibiotics including those commonly used to treat clinical infections such as ampicillin, aztreonam, cefaclor, cephalothin and nalidixic acid (335). Adamu et al. observed differences in antibiotic resistance frequencies in STEC isolates from cattle and camels in Nigeria, with isolates from cattle showing higher resistance frequencies to ampicillin and gentamycin while isolates from camels had higher frequencies of resistance to tetracycline than cattle (336), suggesting that many animals may serve as reservoirs of antibiotic resistant STEC infections.

CURRENT CHALLENGES AND GAPS IN KNOWLEDGE

STEC and NTS are serious public health challenges that contribute to a significant number of food illnesses every year. In addition, the emergence and spread of antibiotic resistance in these pathogens is concerning due to its implications on human health.

Although the FoodNet surveillance system tracks the incidence of foodborne infections in the US, only 15% of the US population is under this surveillance system. Michigan is not part of the FoodNet surveillance system, thus, the incidence of food infections and epidemiological information associated with these cases is not available for Michigan. In addition, there is a dearth in studies looking at frequencies of antibiotic resistance to important antimicrobials and epidemiological factors associated with antibiotic resistant infections in Michigan. While NARMS does monitor frequencies of antibiotic resistance in important pathogens in the US, it does not test non-O157 STEC isolates for antibiotic resistance. Considering the increasing importance of non-O157 as a pathogen, it is crucial to monitor resistance frequencies in these serotypes as well. Over the years, different risk factors have been identified for antibiotic resistant NTS infections, while no studies have been conducted that have attempted to identify risk factors of antibiotic resistant STEC infections and make comparisons between resistance frequencies from cattle- and human-derived strains.

A thorough review of the literature has uncovered geographic variability in risk factors associated with resistant infections, which may be influenced by human behaviour (337-339), environmental factors (340), pathogen factors (257, 340-342) or other variables (343). Indeed, high frequencies of antibiotic resistance were observed in European countries with high outpatient antibiotic use (254). In Michigan, 918-1016 antibiotic prescriptions per 1000 population was

reported in 2015 (344), and this high antibiotic use is likely to be a driving force behind the emergence of antibiotic resistance. The diversity of studies examining antibiotic resistance, differences in study design and sample sizes may also affect results and interpretation of data. Consequently, determining frequencies of antibiotic resistance in isolates from humans and animals, and elucidating factors associated with resistance is crucial in the develop regulatory policies to combat the emergence and spread of resistance worldwide (345, 346). Given the lack of data and knowledge about resistance in foodborne pathogens recovered from Michigan, it is important to quantify resistance frequencies and identify risk factors associated with resistant infections in order to make comparisons to data generated nationally and elsewhere in the world.

It is critical to study the dissemination of resistant clones and genes responsible for drug resistance because of the constant evolution of foodborne pathogens and ease of acquisition of antibiotic resistance genes (ARGs). While studies have looked at the occurrence of antibiotic resistance genes (ARGs) in wastewaters, rivers and in other sources in Michigan (347-349), the diversity and abundance of ARGs in important food pathogens is severely lacking. Although studies in other pathogens have noted correlations between antibiotic resistant mutations and strain diversity (257), there is limited information about associations between phylogenetic lineages of NTS and STEC and resistance. Furthermore, identifying which ARGs and mutations are responsible for resistance will also aid in rapid drug resistance detection techniques.

To address these knowledge gaps, this study was undertaken with the following objectives:

1. Determine the frequency and epidemiological factors associated with clinical antibiotic resistant Shiga toxin-producing *Escherichia coli* (STEC) infections in Michigan, 2010-2014.

Hypotheses: *The frequencies of antibiotic resistance observed in STEC O157 in Michigan are comparable to what is reported for the US by NARMS, and specific geographical, temporal, and molecular factors are associated with resistant STEC*. Furthermore, *antibiotic resistant STEC are more likely to be associated with negative patient outcomes.*

2. Identify trends of antibiotic resistance in Shiga toxin-producing *Escherichia coli* (STEC) infections in Michigan (2001-2014) and factors associated with antibiotic resistant infections.

Hypotheses: *Similar trends in antibiotic resistance frequencies in STEC O157 from Michigan and those tested by NARMS will be observed*. Additionally, *variations in frequencies by serotypes are likely to be observed*.

3. Characterize the genetic determinants of antibiotic resistance in non-O157 serotypes of Shiga toxin-producing *Escherichia coli* (STEC) and association with phylogenetic lineages.

Hypotheses: *Specific genes are important for resistance to antibiotics and multidrug resistance is likely to occur due to the presence of co-occurring ARGs.* Additionally, *resistant non-O157 isolates are likely to belong to unique phylogenetic lineages*.

4. Determine antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* (STEC) recovered from cattle in Michigan.

Hypotheses: *Due to the use and persistence of clinically and veterinary relevant antibiotics in agriculture, antimicrobial resistant STEC are highly abundant in cattle* and further, *that specific herd and farm management practices will be associated with resistant STEC in specific herds*.

5. Determine the frequency and epidemiological factors associated with clinical antibiotic resistant non-typhoidal *Salmonella* (NTS) in Michigan, 2011-2014.

Hypotheses: *The frequencies of antibiotic resistance observed in NTS in Michigan are comparable to what is reported by NARMS for the US*. Furthermore, *specific geographical, temporal, molecular factors and clinical factors are likely to be associated with resistant NTS*.

Overall, this study will enhance our understanding on the molecular epidemiology of antibiotic resistant STEC and NTS infections in Michigan, which is critical for the development of targeted intervention and case management strategies for combatting drug resistant infections.

APPENDIX

Figure 1.1. Classification of the genus *Salmonella*. Adapted from (119, 120)

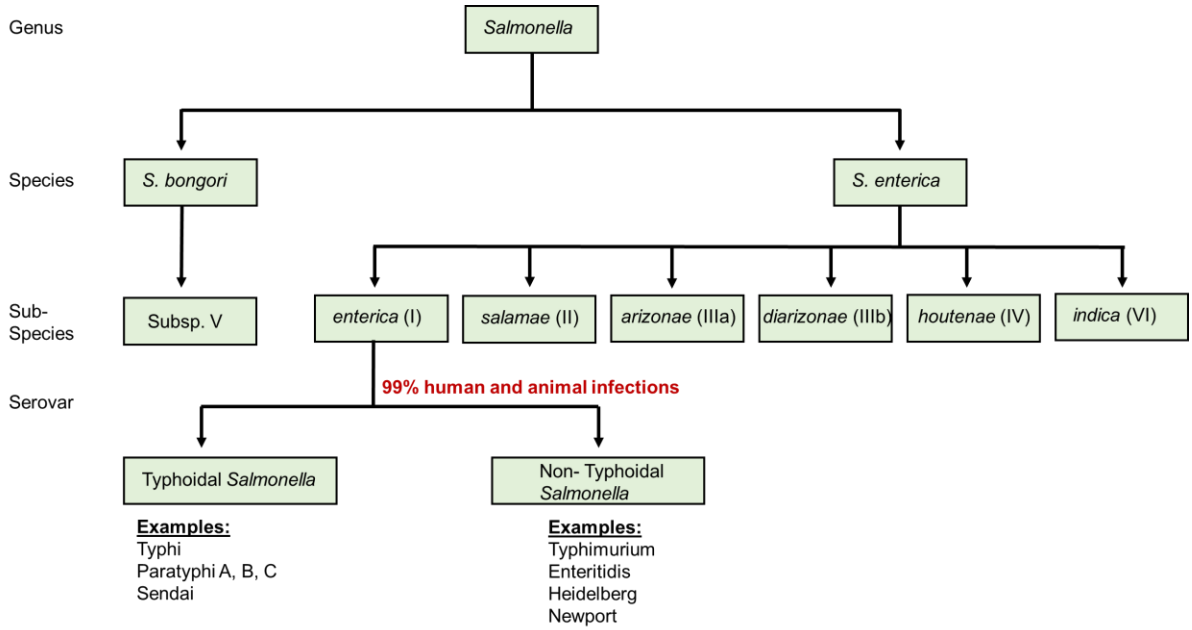
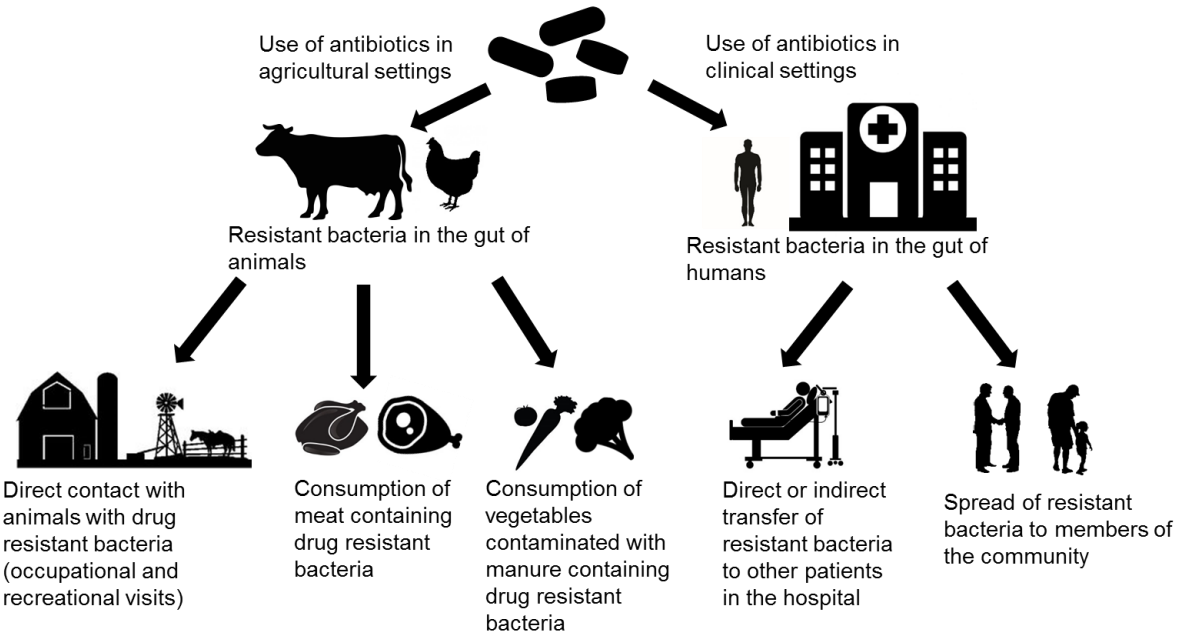


Figure 1.2. Sources of antibiotic resistant organisms. Spread of resistant bacteria may occur via person-person transmission, consumption of contaminated food and water, direct contact with farm animals. Information compiled from CDC: Antibiotic Resistance (191)



REFERENCES

REFERENCES

1. World Health Organization. The global burden of disease: 2004 update. Geneva: World Health Organization. 2008.

2. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B for the World Health Organization Foodborne Disease Burden Epidemiology Reference Group. World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. PLoS Med. 2015;12(12):e1001923. doi: 10.1371/journal.pmed.1001923.

3. Hoffmann S, Batz MB, Morris JG, Jr. Annual cost of illness and quality-adjusted life year losses in the United States due to 14 foodborne pathogens. J Food Prot. 2012;75(7):1292-302. doi: 10.4315/0362-028X.JFP-11-417.

4. Batz M, Hoffmann S, Morris JG, Jr. Disease-outcome trees, EQ-5D scores, and estimated annual losses of quality-adjusted life years (QALYs) for 14 foodborne pathogens in the United States. Foodborne Pathog Dis. 2014;11(5):395-402. doi: 10.1089/fpd.2013.1658.

5. Centers for Disease Control and Prevention. Foodborne Diseases Active Surveillance Network (FoodNet) 2018.

6. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, Davis BR, Hebert RJ, Olcott ES, Johnson LM, Hargrett NT, Blake PA, Cohen ML. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med. 1983;308(12):681-5. doi: 10.1056/NEJM198303243081203.

7. Scheutz F. Taxonomy Meets Public Health: The Case of Shiga Toxin-Producing *Escherichia coli*. Microbiol Spectr. 2014;2(3). doi: 10.1128/microbiolspec.EHEC-0019-2013.

8. Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD. Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. Foodborne Pathog Dis. 2014;11(6):447-55. doi: 10.1089/fpd.2013.1704.

9. Tseng M, Sha Q, Rudrik JT, Collins J, Henderson T, Funk JA, Manning SD. Increasing incidence of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in Michigan and association with clinical illness. Epidemiol Infect. 2016;144(7):1394-405. doi: 10.1017/S0950268815002836.

10. Gould LH, Mody RK, Ong KL, Clogher P, Cronquist AB, Garman KN, Lathrop S, Medus C, Spina NL, Webb TH, White PL, Wymore K, Gierke RE, Mahon BE, Griffin

PM for Emerging Infections Program Foodnet Working Group. Increased recognition of non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States during 2000-2010: epidemiologic features and comparison with *E. coli* O157 infections. Foodborne Pathog Dis. 2013;10(5):453-60. doi: 10.1089/fpd.2012.1401.

11. Bettelheim KA. The non-O157 shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. Crit Rev Microbiol. 2007;33(1):67-87. doi: 10.1080/10408410601172172.

12. Centers for Disease Control and Prevention. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). US Department of Health and Human Services, CDC. 2017.

13. Thorpe CM. Shiga toxin-producing *Escherichia coli* infection. Clin Infect Dis. 2004;38(9):1298-303. doi: 10.1086/383473.

14. Etcheverria AI, Padola NL. Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization. Virulence. 2013;4(5):366-72. doi: 10.4161/viru.24642.

15. Canpolat N. Hemolytic uremic syndrome. Turk Pediatri Ars. 2015;50(2):73-82. doi: 10.5152/tpa.2015.2297.

16. Siegler RL. The Hemolytic Uremic Syndrome. Pediatric Clinics of North America. 1995;42(6):1505-29. doi: https://doi.org/10.1016/S0031-3955(16)40096-9.

17. Vygen-Bonnet S, Rosner B, Wilking H, Fruth A, Prager R, Kossow A, Lang C, Simon S, Seidel J, Faber M, Schielke A, Michaelis K, Holzer A, Kamphausen R, Kalhofer D, Thole S, Mellmann A, Flieger A, Stark K. Ongoing haemolytic uraemic syndrome (HUS) outbreak caused by sorbitol-fermenting (SF) Shiga toxin-producing *Escherichia coli* (STEC) O157, Germany, December 2016 to May 2017. Euro Surveill. 2017;22(21). doi: 10.2807/1560-7917.ES.2017.22.21.30541.

18. Tozzi AE, Caprioli A, Minelli F, Gianviti A, De Petris L, Edefonti A, Montini G, Ferretti A, De Palo T, Gaido M, Rizzoni G, and the Hemolytic Uremic Syndrome Study Group. Shiga toxin-producing *Escherichia coli* infections associated with hemolytic uremic syndrome, Italy, 1988-2000. Emerg Infect Dis. 2003;9(1):106-8. doi: 10.3201/eid0901.020266.

19. Gould LH, Demma L, Jones TF, Hurd S, Vugia DJ, Smith K, Shiferaw B, Segler S, Palmer A, Zansky S, Griffin PM. Hemolytic uremic syndrome and death in persons with *Escherichia coli* O157:H7 infection, foodborne diseases active surveillance network sites, 2000-2006. Clin Infect Dis. 2009;49(10):1480-5. doi: 10.1086/644621.

20. Loos S, Ahlenstiel T, Kranz B, Staude H, Pape L, Hartel C, Vester U, Buchtala L, Benz K, Hoppe B, Beringer O, Krause M, Muller D, Pohl M, Lemke J, Hillebrand G, Kreuzer M, Konig J, Wigger M, Konrad M, Haffner D, Oh J, Kemper MJ. An outbreak of Shiga

toxin-producing *Escherichia coli* O104:H4 hemolytic uremic syndrome in Germany: presentation and short-term outcome in children. Clin Infect Dis. 2012;55(6):753-9. doi: 10.1093/cid/cis531.

21. O'Brien AD, LaVeck GD, Thompson MR, Formal SB. Production of *Shigella dysenteriae* type 1-like cytotoxin by Escherichia coli. J Infect Dis. 1982;146(6):763-9.

22. O'Brien AD, LaVeck GD. Purification and characterization of a *Shigella dysenteriae* 1-like toxin produced by *Escherichia coli*. Infect Immun. 1983;40(2):675-83.

23. Cleary TG, Mathewson JJ, Faris E, Pickering LK. Shiga-like cytotoxin production by enteropathogenic *Escherichia coli* serogroups. Infect Immun. 1985;47(1):335-7.

24. Melton-Celsa AR. Shiga Toxin (Stx) Classification, Structure, and Function. Microbiology spectrum. 2014;2(2):10.1128/microbiolspec.EHEC-0024-2013. doi: 10.1128/microbiolspec.EHEC-0024-2013.

25. Boyd B, Lingwood C. Verotoxin receptor glycolipid in human renal tissue. Nephron. 1989;51(2):207-10. doi: 10.1159/000185286.

26. Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S, Karmali M. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. J Biol Chem. 1987;262(18):8834-9.

27. Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem. 1988;171(1-2):45-50.

28. O'Brien AD, Holmes RK. Shiga and Shiga-like toxins. Microbiol Rev. 1987;51(2):206-20.

29. Tumer NE, Li XP. Interaction of ricin and Shiga toxins with ribosomes. Curr Top Microbiol Immunol. 2012;357:1-18. doi: 10.1007/82_2011_174.

30. Walsh MJ, Dodd JE, Hautbergue GM. Ribosome-inactivating proteins: potent poisons and molecular tools. Virulence. 2013;4(8):774-84. doi: 10.4161/viru.26399.

31. Grisaru S. Management of hemolytic-uremic syndrome in children. Int J Nephrol Renovasc Dis. 2014;7:231-9. doi: 10.2147/IJNRD.S41837.

32. Obrig TG, Karpman D. Shiga toxin pathogenesis: kidney complications and renal failure. Curr Top Microbiol Immunol. 2012;357:105-36. doi: 10.1007/82_2011_172.

33. Konowalchuk J, Speirs JI, Stavric S. Vero response to a cytotoxin of *Escherichia coli*. Infect Immun. 1977;18(3):775-9. Epub 1977/12/01.

34. O'Brien AO, Lively TA, Chen ME, Rothman SW, Formal SB. *Escherichia coli* O157:H7 strains associated with haemorrhagic colitis in the United States produce a *Shigella dysenteriae* 1 (SHIGA) like cytotoxin. Lancet. 1983;1(8326 Pt 1):702.

35. Smith HR, Scotland SM. Vero cytotoxin-producing strains of *Escherichia coli*. J Med Microbiol. 1988;26(2):77-85. doi: 10.1099/00222615-26-2-77.

36. Strockbine NA, Marques LR, Newland JW, Smith HW, Holmes RK, O'Brien AD. Two toxin-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. Infect Immun. 1986;53(1):135-40.

37. Jackson MP, Newland JW, Holmes RK, O'Brien AD. Nucleotide sequence analysis of the structural genes for Shiga-like toxin I encoded by bacteriophage 933J from *Escherichia coli*. Microb Pathog. 1987;2(2):147-53.

38. Golshani M, Oloomi M, Bouzari S. In silico analysis of Shiga toxins (Stxs) to identify new potential vaccine targets for Shiga toxin-producing *Escherichia coli*. In Silico Pharmacol. 2016;5(1):2. doi: 10.1007/s40203-017-0022-4.

39. Paton JC, Paton AW. Pathogenesis and Diagnosis of Shiga Toxin-Producing *Escherichia coli* Infections. Clinical Microbiology Reviews. 1998;11(3):450-79.

40. Schuller S. Shiga toxin interaction with human intestinal epithelium. Toxins (Basel). 2011;3(6):626-39. doi: 10.3390/toxins3060626.

41. Hurley BP, Thorpe CM, Acheson DW. Shiga toxin translocation across intestinal epithelial cells is enhanced by neutrophil transmigration. Infect Immun. 2001;69(10):6148-55. doi: 10.1128/IAI.69.10.6148-6155.2001.

42. Hurley BP, Jacewicz M, Thorpe CM, Lincicome LL, King AJ, Keusch GT, Acheson DW. Shiga toxins 1 and 2 translocate differently across polarized intestinal epithelial cells. Infect Immun. 1999;67(12):6670-7.

43. Kruger A, Lucchesi PM. Shiga toxins and stx phages: highly diverse entities. Microbiology. 2015;161(Pt 3):451-62. doi: 10.1099/mic.0.000003.

44. O'Brien AD, Newland JW, Miller SF, Holmes RK, Smith HW, Formal SB. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhea. Science. 1984;226(4675):694-6.

45. Gyles CL. Shiga toxin-producing *Escherichia coli*: an overview. J Anim Sci. 2007;85(13 Suppl):E45-62. doi: 10.2527/jas.2006-508.

46. Herold S, Karch H, Schmidt H. Shiga toxin-encoding bacteriophages--genomes in motion. Int J Med Microbiol. 2004;294(2-3):115-21. doi: 10.1016/j.ijmm.2004.06.023.

47. Wagner PL, Neely MN, Zhang X, Acheson DW, Waldor MK, Friedman DI. Role for a phage promoter in Shiga toxin 2 expression from a pathogenic *Escherichia coli* strain. J Bacteriol. 2001;183(6):2081-5. doi: 10.1128/JB.183.6.2081-2085.2001.

48. Wagner PL, Livny J, Neely MN, Acheson DW, Friedman DI, Waldor MK. Bacteriophage control of Shiga toxin 1 production and release by *Escherichia coli*. Mol Microbiol. 2002;44(4):957-70.

49. Kimmitt PT, Harwood CR, Barer MR. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. Emerg Infect Dis. 2000;6(5):458-65. doi: 10.3201/eid0605.000503.

50. Muniesa M, Serra-Moreno R, Jofre J. Free Shiga toxin bacteriophages isolated from sewage showed diversity although the stx genes appeared conserved. Environ Microbiol. 2004;6(7):716-25. doi: 10.1111/j.1462-2920.2004.00604.x.

51. Dumke R, Schroter-Bobsin U, Jacobs E, Roske I. Detection of phages carrying the Shiga toxin 1 and 2 genes in waste water and river water samples. Lett Appl Microbiol. 2006;42(1):48-53. doi: 10.1111/j.1472-765X.2005.01809.x.

52. Oot RA, Raya RR, Callaway TR, Edrington TS, Kutter EM, Brabban AD. Prevalence of *Escherichia coli* O157 and O157:H7-infecting bacteriophages in feedlot cattle feces. Lett Appl Microbiol. 2007;45(4):445-53. doi: 10.1111/j.1472-765X.2007.02211.x.

53. Muniesa M, Hammerl JA, Hertwig S, Appel B, Brüssow H. Shiga Toxin-Producing *Escherichia coli* O104:H4: a New Challenge for Microbiology. Applied and Environmental Microbiology. 2012;78(12):4065-73. doi: 10.1128/AEM.00217-12.

54. Rasko DA, Webster DR, Sahl JW, Bashir A, Boisen N, Scheutz F, Paxinos EE, Sebra R, Chin C-S, Iliopoulos D, Klammer A, Peluso P, Lee L, Kislyuk AO, Bullard J, Kasarskis A, Wang S, Eid J, Rank D, Redman JC, Steyert SR, Frimodt-Møller J, Struve C, Petersen AM, Krogfelt KA, Nataro JP, Schadt EE, Waldor MK. Origins of the *E. coli* Strain Causing an Outbreak of Hemolytic–Uremic Syndrome in Germany. The New England journal of medicine. 2011;365(8):709-17. doi: 10.1056/NEJMoa1106920.

55. Yu J, Kaper JB. Cloning and characterization of the eae gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol Microbiol. 1992;6(3):411-7. doi: 10.1111/j.1365-2958.1992.tb01484.x.

56. Dean-Nystrom EA, Bosworth BT, Moon HW, O'Brien AD. *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. Infect Immun. 1998;66(9):4560-3.

57. Frankel G, Phillips AD, Trabulsi LR, Knutton S, Dougan G, Matthews S. Intimin and the host cell--is it bound to end in Tir(s)? Trends Microbiol. 2001;9(5):214-8.

58. Schmidt H, Beutin L, Karch H. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. Infect Immun. 1995;63(3):1055-61.

59. Paton AW, Srimanote P, Woodrow MC, Paton JC. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. Infect Immun. 2001;69(11):6999-7009. doi: 10.1128/IAI.69.11.6999-7009.2001.

60. Benjamin MM, Datta AR. Acid tolerance of enterohemorrhagic *Escherichia coli*. Appl Environ Microbiol. 1995;61(4):1669-72.

61. Waterman SR, Small PL. Acid-sensitive enteric pathogens are protected from killing under extremely acidic conditions of pH 2.5 when they are inoculated onto certain solid food sources. Appl Environ Microbiol. 1998;64(10):3882-6.

62. Waterman SR, Small PL. Characterization of the acid resistance phenotype and *rpoS* alleles of shiga-like toxin-producing *Escherichia coli*. Infect Immun. 1996;64(7):2808-11.

63. Lin J, Smith MP, Chapin KC, Baik HS, Bennett GN, Foster JW. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl Environ Microbiol. 1996;62(9):3094-100.

64. Castro VS, Carvalho RCT, Conte-Junior CA, Figuiredo EES. Shiga-toxin Producing *Escherichia coli*: Pathogenicity, Supershedding, Diagnostic Methods, Occurrence, and Foodborne Outbreaks. Comprehensive Reviews in Food Science and Food Safety. 2017;16(6):1269-80. doi: doi:10.1111/1541-4337.12302.

65. Zschock M, Hamann HP, Kloppert B, Wolter W. Shiga-toxin-producing *Escherichia coli* in faeces of healthy dairy cows, sheep and goats: prevalence and virulence properties. Lett Appl Microbiol. 2000;31(3):203-8.

66. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J Clin Microbiol. 1993;31(9):2483-8.

67. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvirta T, Pelkonen S, Yamamoto K. Prevalence and characteristics of shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl Environ Microbiol. 2001;67(1):484-9. doi: 10.1128/AEM.67.1.484-489.2001.

68. Hussein HS, Bollinger LM. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. J Food Prot. 2005;68(10):2224-41.

69. Cobbold RN, Rice DH, Szymanski M, Call DR, Hancock DD. Comparison of shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in

Washington State. Appl Environ Microbiol. 2004;70(7):4375-8. doi: 10.1128/AEM.70.7.4375-4378.2004.

70. Cobbold R, Desmarchelier P. A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian diary herds. Vet Microbiol. 2000;71(1-2):125-37.

71. Geue L, Segura-Alvarez M, Conraths FJ, Kuczius T, Bockemuhl J, Karch H, Gallien P. A long-term study on the prevalence of shiga toxin-producing *Escherichia coli* (STEC) on four German cattle farms. Epidemiol Infect. 2002;129(1):173-85.

72. Wells JG, Shipman LD, Greene KD, Sowers EG, Green JH, Cameron DN, Downes FP, Martin ML, Griffin PM, Ostroff SM, et al. Isolation of *Escherichia coli* serotype O157:H7 and other Shiga-like-toxin-producing *E. coli* from dairy cattle. J Clin Microbiol. 1991;29(5):985-9.

73. Cernicchiaro N, Pearl DL, Ghimire S, Gyles CL, Johnson RP, LeJeune JT, Ziebell K, McEwen SA. Risk factors associated with *Escherichia coli* O157:H7 in Ontario beef cow-calf operations. Prev Vet Med. 2009;92(1-2):106-15. doi: 10.1016/j.prevetmed.2009.07.004.

74. Venegas-Vargas C, Henderson S, Khare A, Mosci RE, Lehnert JD, Singh P, Ouellette LM, Norby B, Funk JA, Rust S, Bartlett PC, Grooms D, Manning SD. Factors Associated with Shiga Toxin-Producing *Escherichia coli* Shedding by Dairy and Beef Cattle. Appl Environ Microbiol. 2016;82(16):5049-56. doi: 10.1128/AEM.00829-16.

75. Chase-Topping M, Gally D, Low C, Matthews L, Woolhouse M. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. Nat Rev Microbiol. 2008;6(12):904-12. doi: 10.1038/nrmicro2029.

76. Cho S, Fossler CP, Diez-Gonzalez F, Wells SJ, Hedberg CW, Kaneene JB, Ruegg PL, Warnick LD, Bender JB. Cattle-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria on dairy farms, Minnesota, USA. Can J Vet Res. 2009;73(2):151-6.

77. Brown CA, Harmon BG, Zhao T, Doyle MP. Experimental *Escherichia coli* O157:H7 carriage in calves. Appl Environ Microbiol. 1997;63(1):27-32.

78. Walker C, Shi X, Sanderson M, Sargeant J, Nagaraja TG. Prevalence of *Escherichia coli* O157:H7 in gut contents of beef cattle at slaughter. Foodborne Pathog Dis. 2010;7(3):249-55. doi: 10.1089/fpd.2009.0410.

79. Keen JE, Laegreid WW, Chitko-McKown CG, Durso LM, Bono JL. Distribution of Shiga-Toxigenic *Escherichia coli* O157 in the Gastrointestinal Tract of Naturally O157-Shedding Cattle at Necropsy. Appl Environ Microbiol 2010;76(15):5278-81. doi: 10.1128/AEM.00400-10.

80. Grauke LJ, Kudva IT, Yoon JW, Hunt CW, Williams CJ, Hovde CJ. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. Appl Environ Microbiol. 2002;68(5):2269-77.

81. Pruimboom-Brees IM, Morgan TW, Ackermann MR, Nystrom ED, Samuel JE, Cornick NA, Moon HW. Cattle lack vascular receptors for *Escherichia coli* O157:H7 Shiga toxins. Proc Natl Acad Sci U S A. 2000;97(19):10325-9. doi: 10.1073/pnas.190329997.

82. Schoonderwoerd M, Clarke RC, van Dreumel AA, Rawluk SA. Colitis in calves: natural and experimental infection with a verotoxin-producing strain of *Escherichia coli* O111:NM. Can J Vet Res. 1988;52(4):484-7.

83. Hale CR, Scallan E, Cronquist AB, Dunn J, Smith K, Robinson T, Lathrop S, Tobin-D'Angelo M, Clogher P. Estimates of enteric illness attributable to contact with animals and their environments in the United States. Clin Infect Dis. 2012;54 Suppl 5:S472-9. doi: 10.1093/cid/cis051.

84. Mughini-Gras L, van Pelt W, van der Voort M, Heck M, Friesema I, Franz E. Attribution of human infections with Shiga toxin-producing *Escherichia coli* (STEC) to livestock sources and identification of source-specific risk factors, The Netherlands (2010-2014). Zoonoses Public Health. 2018;65(1):e8-e22. doi: 10.1111/zph.12403.

85. Schlager S, Lepuschitz S, Ruppitsch W, Ableitner O, Pietzka A, Neubauer S, Stöger A, Lassnig H, Mikula C, Springer B, Allerberger F. Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. Int J Med Microbiol. 2018; 308(7):927-932. doi: https://doi.org/10.1016/j.ijmm.2018.06.008.

86. Goode B, O'Reilly C, Dunn J, et al. Outbreak of *Escherichia coli* O157: H7 infections after petting zoo visits, north carolina state fair, october-november 2004. Arch Pediatr Adolesc Med. 2009;163(1):42-8. doi: 10.1001/archpediatrics.2008.525.

87. Centers for Disease Control and Prevention. Outbreak of Shiga toxin-producing *Escherichia coli* O157 infection associated with a day camp petting zoo--Pinellas County, Florida, May-June 2007. MMWR Morb Mortal Wkly Rep. 2009;58(16):426-8.

88. Brusa V, Aliverti V, Aliverti F, Ortega EE, de la Torre JH, Linares LH, Sanz ME, Etcheverria AI, Padola NL, Galli L, Peral Garcia P, Copes J, Leotta GA. Shiga toxin-producing *Escherichia coli* in beef retail markets from Argentina. Front Cell Infect Microbiol. 2012;2:171. doi: 10.3389/fcimb.2012.00171.

89. Hussein HS. Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. J Anim Sci. 2007;85(13 Suppl):E63-72. doi: 10.2527/jas.2006-421.

90. Centers for Disease Control and Prevention. Reports of Selected *E. coli* Outbreak Investigations. 2018.

91. Guh A, Phan Q, Nelson R, Purviance K, Milardo E, Kinney S, Mshar P, Kasacek W, Cartter M. Outbreak of *Escherichia coli* O157 associated with raw milk, Connecticut, 2008. Clin Infect Dis. 2010;51(12):1411-7. doi: 10.1086/657304.

92. Mohammadi P, Abiri R, Rezaei M, Salmanzadeh-Ahrabi S. Isolation of Shiga toxin-producing *Escherichia coli* from raw milk in Kermanshah, Iran. Iran J Microbiol. 2013;5(3):233-8.

93. Robinson TJ, Scheftel JM, Smith KE. Raw Milk Consumption among Patients with Non–Outbreak-related Enteric Infections, Minnesota, USA, 2001–2010. Emerging Infectious Diseases. 2014;20(1):38-44. doi: 10.3201/eid2001.120920.

94. Slayton RB, Turabelidze G, Bennett SD, Schwensohn CA, Yaffee AQ, Khan F, Butler C, Trees E, Ayers TL, Davis ML, Laufer AS, Gladbach S, Williams I, Gieraltowski LB. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. PLoS One. 2013;8(2):e55300. doi: 10.1371/journal.pone.0055300.

95. Centers for Disease Control and Prevention. Multistate Outbreak of *E. coli* O157:H7 Infections Linked to Romaine Lettuce (Final Update)2018.

96. Bohnlein C, Kabisch J, Muller-Herbst S, Fiedler G, Franz C, Pichner R. Persistence and reduction of Shiga toxin-producing *Escherichia coli* serotype O26:H11 in different types of raw fermented sausages. Int J Food Microbiol. 2017;261:82-8. doi: 10.1016/j.ijfoodmicro.2017.09.019.

97. MacDonald DM, Fyfe M, Paccagnella A, Trinidad A, Louie K, Patrick D. *Escherichia coli* O157:H7 outbreak linked to salami, British Columbia, Canada, 1999. Epidemiol Infect. 2004;132(2):283-9.

98. Lascowski KM, Guth BE, Martins FH, Rocha SP, Irino K, Pelayo JS. Shiga toxin-producing *Escherichia coli* in drinking water supplies of north Parana State, Brazil. J Appl Microbiol. 2013;114(4):1230-9. doi: 10.1111/jam.12113.

99. Probert WS, Miller GM, Ledin KE. Contaminated Stream Water as Source for *Escherichia coli* O157 Illness in Children. Emerg Infect Dis. 2017;23(7):1216-8. doi: 10.3201/eid2307.170226.

100. Solomon EB, Yaron S, Matthews KR. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl Environ Microbiol. 2002;68(1):397-400.

101. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated

with contaminated manure composts or irrigation water. J Food Prot. 2004;67(7):1365-70.

102. Strachan NJ, Dunn GM, Locking ME, Reid TM, Ogden ID. *Escherichia coli* O157: burger bug or environmental pathogen? Int J Food Microbiol. 2006;112(2):129-37. doi: 10.1016/j.ijfoodmicro.2006.06.021.

103. Werber D, Mason BW, Evans MR, Salmon RL. Preventing household transmission of Shiga toxin-producing *Escherichia coli* O157 infection: promptly separating siblings might be the key. Clin Infect Dis. 2008;46(8):1189-96. doi: 10.1086/587670.

104. Effler E, Isaacson M, Arntzen L, Heenan R, Canter P, Barrett T, Lee L, Mambo C, Levine W, Zaidi A, Griffin PM. Factors contributing to the emergence of *Escherichia coli* O157 in Africa. Emerg Infect Dis. 2001;7(5):812-9. doi: 10.3201/eid0705.017507.

105. Rivas M, Sosa-Estani S, Rangel J, Caletti MG, Valles P, Roldan CD, Balbi L, Marsano de Mollar MC, Amoedo D, Miliwebsky E, Chinen I, Hoekstra RM, Mead P, Griffin PM. Risk factors for sporadic Shiga toxin-producing *Escherichia coli* infections in children, Argentina. Emerg Infect Dis. 2008;14(5):763-71. doi: 10.3201/eid1405.071050.

106. Kassenborg HD, Hedberg CW, Hoekstra M, Evans MC, Chin AE, Marcus R, Vugia DJ, Smith K, Ahuja SD, Slutsker L, Griffin PM, Emerging Infections Program FoodNet Working G. Farm visits and undercooked hamburgers as major risk factors for sporadic *Escherichia coli* O157:H7 infection: data from a case-control study in 5 FoodNet sites. Clin Infect Dis. 2004;38 Suppl 3:S271-8. doi: 10.1086/381596.

107. Voetsch AC, Kennedy MH, Keene WE, Smith KE, Rabatsky-Ehr T, Zansky S, Thomas SM, Mohle-Boetani J, Sparling PH, McGavern MB, Mead PS. Risk factors for sporadic Shiga toxin-producing *Escherichia coli* O157 infections in FoodNet sites, 1999-2000. Epidemiol Infect. 2007;135(6):993-1000. doi: 10.1017/S0950268806007564.

108. Liao YT, Miller MF, Loneragan GH, Brooks JC, Echeverry A, Brashears MM. Non-O157 Shiga toxin-producing *Escherichia coli* in U. S. retail ground beef. J Food Prot. 2014;77(7):1188-92. doi: 10.4315/0362-028X.JFP-13-518.

109. Barlow RS, Gobius KS, Desmarchelier PM. Shiga toxin-producing *Escherichia coli* in ground beef and lamb cuts: results of a one-year study. Int J Food Microbiol. 2006;111(1):1-5. doi: 10.1016/j.ijfoodmicro.2006.04.039.

110. Bosilevac JM, Koohmaraie M. Prevalence and characterization of non-O157 shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. Appl Environ Microbiol. 2011;77(6):2103-12. doi: 10.1128/AEM.02833-10.

111. Centers for Disease Control and Prevention. Multistate Outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 Infections Linked to Ground Beef (Final Update). 2014.

112. McPherson M, Lalor K, Combs B, Raupach J, Stafford R, Kirk MD. Serogroup-specific risk factors for Shiga toxin-producing *Escherichia coli* infection in Australia. Clin Infect Dis. 2009;49(2):249-56. doi: 10.1086/599370.

113. Crump JA, Sulka AC, Langer AJ, Schaben C, Crielly AS, Gage R, Baysinger M, Moll M, Withers G, Toney DM, Hunter SB, Hoekstra RM, Wong SK, Griffin PM, Van Gilder TJ. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. N Engl J Med. 2002;347(8):555-60. doi: 10.1056/NEJMoa020524.

114. Werber D, Behnke SC, Fruth A, Merle R, Menzler S, Glaser S, Kreienbrock L, Prager R, Tschape H, Roggentin P, Bockemuhl J, Ammon A. Shiga toxin-producing *Escherichia coli* infection in Germany: different risk factors for different age groups. Am J Epidemiol. 2007;165(4):425-34. doi: 10.1093/aje/kwk023.

115. Friesema IHM, Schotsborg M, Heck MEOC, Van Pelt W. Risk factors for sporadic Shiga toxin-producing *Escherichia coli* O157 and non-O157 illness in The Netherlands, 2008–2012, using periodically surveyed controls. Epidemiol Infect. 2014;143(7):1360-7. doi: 10.1017/S0950268814002349.

116. Hadler JL, Clogher P, Hurd S, Phan Q, Mandour M, Bemis K, Marcus R. Ten-year trends and risk factors for non-O157 Shiga toxin-producing *Escherichia coli* found through Shiga toxin testing, Connecticut, 2000-2009. Clin Infect Dis. 2011;53(3):269-76. doi: 10.1093/cid/cir377.

117. Karch H, Bielaszewska M, Bitzan M, Schmidt H. Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Diagn Microbiol Infect Dis. 1999;34(3):229-43.

118. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1.

119. Pui CF, Wong WC, Chai LC, Tunung R, Jeyaletchumi P, Noor Hidayah MS, Ubong A, Farinazleen MG, Cheah YK, Son R. *Salmonella* : A foodborne pathogen. Int Food Res J. 2011;473:465–47.

120. Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. *Salmonella* nomenclature. J Clin Microbiol. 2000;38(7):2465-7.

121. Gal-Mor O, Boyle EC, Grassl GA. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. Front Microbiol. 2014;5:391. doi: 10.3389/fmicb.2014.00391.

122. Hendriksen RS, Vieira AR, Karlsmose S, Lo Fo Wong DM, Jensen AB, Wegener HC, Aarestrup FM. Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of

quality assured laboratories from 2001 to 2007. Foodborne Pathog Dis. 2011;8(8):887-900. doi: 10.1089/fpd.2010.0787.

123. Andino A, Hanning I. *Salmonella enterica*: survival, colonization, and virulence differences among serovars. ScientificWorldJournal. 2015;2015:520179. doi: 10.1155/2015/520179.

124. Foster JW. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J Bacteriol. 1991;173(21):6896-902. doi: 10.1128/jb.173.21.6896-6902.1991.

125. Wilmes-Riesenberg MR, Bearson B, Foster JW, Curtis R, 3rd. Role of the acid tolerance response in virulence of *Salmonella* Typhimurium. Infect Immun. 1996;64(4):1085-92.

126. Alvarez-Ordonez A, Begley M, Prieto M, Messens W, Lopez M, Bernardo A, Hill C. *Salmonella* spp. survival strategies within the host gastrointestinal tract. Microbiology. 2011;157(Pt 12):3268-81. doi: 10.1099/mic.0.050351-0.

127. Hurley D, McCusker MP, Fanning S, Martins M. *Salmonella*-host interactions - modulation of the host innate immune system. Front Immunol. 2014;5:481. doi: 10.3389/fimmu.2014.00481.

128. Ramos-Morales F. Impact of *Salmonella enterica* Type III Secretion System Effectors on the Eukaryotic Host Cell. ISRN Cell Biology. 2012;2012:36. doi: 10.5402/2012/787934.

129. Swart AL, Hensel M. Interactions of *Salmonella enterica* with dendritic cells. Virulence. 2012;3(7):660-7. doi: 10.4161/viru.22761.

130. Jepson MA, Clark MA. The role of M cells in *Salmonella* infection. Microbes Infect. 2001;3(14-15):1183-90.

131. Steele-Mortimer O. The *Salmonella*-containing Vacuole – Moving with the Times. Curr Opin Microbiol. 2008;11(1):38-45. doi: 10.1016/j.mib.2008.01.002.

132. Chen HM, Wang Y, Su LH, Chiu CH. Nontyphoid *Salmonella* infection: microbiology, clinical features, and antimicrobial therapy. Pediatr Neonatol. 2013;54(3):147-52. doi: 10.1016/j.pedneo.2013.01.010.

133. Blaser MJ, Newman LS. A review of human salmonellosis: I. Infective dose. Rev Infect Dis. 1982;4(6):1096-106.

134. Glynn JR, Palmer SR. Incubation period, severity of disease, and infecting dose: evidence from a *Salmonella* outbreak. Am J Epidemiol. 1992;136(11):1369-77.

135. Acheson D, Hohmann EL. Nontyphoidal Salmonellosis. Clin Infect Dis. 2001;32(2):263-9. doi: 10.1086/318457.

136. Trong TA, Nicholas AF, Melita AG, Karen HK, Frederick JA, John AC. Global Burden of Invasive Nontyphoidal Salmonella Disease, 2010. Emerg Infect Dis. 2015;21(6):941. doi: 10.3201/eid2106.140999.

137. Uzzau S, Brown DJ, Wallis T, Rubino S, Leori G, Bernard S, Casadesus J, Platt DJ, Olsen JE. Host adapted serotypes of *Salmonella enterica*. Epidemiol Infect. 2000;125(2):229-55.

138. Chao MR, Hsien CH, Yeh CM, Chou SJ, Chu C, Su YC, Yu CY. Assessing the prevalence of *Salmonella enterica* in poultry hatcheries by using hatched eggshell membranes. Poult Sci. 2007;86(8):1651-5. doi: 10.1093/ps/86.8.1651.

139. Zhao X, Gao Y, Ye C, Yang L, Wang T, Chang W. Prevalence and Characteristics of *Salmonella* Isolated from Free-Range Chickens in Shandong Province, China. Biomed Res Int. 2016;2016:8183931. doi: 10.1155/2016/8183931.

140. Donado-Godoy P, Clavijo V, Leon M, Tafur MA, Gonzales S, Hume M, Alali W, Walls I, Lo Fo Wong DM, Doyle MP. Prevalence of *Salmonella* on retail broiler chicken meat carcasses in Colombia. J Food Prot. 2012;75(6):1134-8. doi: 10.4315/0362-028X.JFP-11-513.

141. Singh S, Yadav AS, Singh SM, Bharti P. Prevalence of *Salmonella* in chicken eggs collected from poultry farms and marketing channels and their antimicrobial resistance. Food Res Int. 2010;43(8):2027-30. doi: https://doi.org/10.1016/j.foodres.2010.06.001.

142. Nidaullah H, Abirami N, Shamila-Syuhada AK, Chuah LO, Nurul H, Tan TP, Abidin FWZ, Rusul G. Prevalence of *Salmonella* in poultry processing environments in wet markets in Penang and Perlis, Malaysia. Vet World. 2017;10(3):286-92. doi: 10.14202/vetworld.2017.286-292.

143. Obukhovska O. The Natural Reservoirs of *Salmonella* Enteritidis in Populations of Wild Birds. Online J Public Health Inform. 2013;5(1):e171.

144. Sorensen O, McFall M, Manninen K. Prevalence of *Salmonella* in dairy herds in Alberta. Can Vet J. 2003;44(3):230-1.

145. Blau DM, McCluskey BJ, Ladely SR, Dargatz DA, Fedorka-Cray PJ, Ferris KE, Headrick ML. *Salmonella* in dairy operations in the United States: prevalence and antimicrobial drug susceptibility. J Food Prot. 2005;68(4):696-702.

146. Mainar-Jaime RC, Atashparvar N, Chirino-Trejo M, Rahn K. Survey on *Salmonella* prevalence in slaughter pigs from Saskatchewan. Can Vet J. 2008;49(8):793-6.

147. Futagawa-Saito K, Hiratsuka S, Kamibeppu M, Hirosawa T, Oyabu K, Fukuyasu T. *Salmonella* in healthy pigs: prevalence, serotype diversity and antimicrobial resistance

observed during 1998-1999 and 2004-2005 in Japan. Epidemiol Infect. 2008;136(8):1118-23. doi: 10.1017/S0950268807009570.

148. Alpigiani I, Bacci C, Lanzoni E, Brindani F, Bonardi S. *Salmonella enterica* Prevalence in Finishing Pigs at Slaughter Plants in Northern Italy. Ital J Food Saf. 2014;3(2):1609. doi: 10.4081/ijfs.2014.1609.

149. Hoelzer K, Moreno Switt AI, Wiedmann M. Animal contact as a source of human non-typhoidal salmonellosis. Vet Res. 2011;42(1):34-. doi: 10.1186/1297-9716-42-34.

150. Fone DL, Barker RM. Associations between human and farm animal infections with *Salmonella* Typhimurium DT104 in Herefordshire. Commun Dis Rep CDR Rev. 1994;4(11):R136-40.

151. Hendriksen SW, Orsel K, Wagenaar JA, Miko A, van Duijkeren E. Animal-to-human transmission of *Salmonella* Typhimurium DT104A variant. Emerg Infect Dis. 2004;10(12):2225-7. doi: 10.3201/eid1012.040286.

152. Leonard F. *Salmonella* infection and carriage: the importance of dogs and their owners. Vet Rec. 2014;174(4):92-3. doi: 10.1136/vr.g367.

153. Centers for Disease Control and Prevention. Reports of Selected *Salmonella* Outbreak Investigations. 2018.

154. Hanning IB, Nutt JD, Ricke SC. Salmonellosis outbreaks in the United States due to fresh produce: sources and potential intervention measures. Foodborne Pathog Dis. 2009;6(6):635-48. doi: 10.1089/fpd.2008.0232.

155. Tournas VH. Spoilage of vegetable crops by bacteria and fungi and related health hazards. Crit Rev Microbiol. 2005;31(1):33-44. doi: 10.1080/10408410590886024.

156. Levantesi C, Bonadonna L, Briancesco R, Grohmann E, Toze S, Tandoi V. *Salmonella* in surface and drinking water: Occurrence and water-mediated transmission. Food Res Int. 2012;45(2):587-602. doi: https://doi.org/10.1016/j.foodres.2011.06.037.

157. Gast RK, Mitchell BW, Holt PS. Airborne transmission of *Salmonella* Enteritidis infection between groups of chicks in controlled-environment isolation cabinets. Avian Dis. 1998;42(2):315-20.

158. Afema JA, Byarugaba DK, Shah DH, Atukwase E, Nambi M, Sischo WM. Potential Sources and Transmission of *Salmonella* and Antimicrobial Resistance in Kampala, Uganda. PLoS One. 2016;11(3):e0152130. doi: 10.1371/journal.pone.0152130.

159. Odoch T, Wasteson Y, L'Abee-Lund T, Muwonge A, Kankya C, Nyakarahuka L, Tegule S, Skjerve E. Prevalence, antimicrobial susceptibility and risk factors associated with

non-typhoidal *Salmonella* on Ugandan layer hen farms. BMC Vet Res. 2017;13(1):365. doi: 10.1186/s12917-017-1291-1.

160. Mughini-Gras L, Enserink R, Friesema I, Heck M, van Duynhoven Y, van Pelt W. Risk factors for human salmonellosis originating from pigs, cattle, broiler chickens and egg laying hens: a combined case-control and source attribution analysis. PLoS One. 2014;9(2):e87933. doi: 10.1371/journal.pone.0087933.

161. Stam F, Romkens TE, Hekker TA, Smulders YM. Turtle-associated human salmonellosis. Clin Infect Dis. 2003;37(11):e167-9. doi: 10.1086/379612.

162. Mermin J, Hutwagner L, Vugia D, Shallow S, Daily P, Bender J, Koehler J, Marcus R, Angulo FJ, Emerging Infections Program FoodNet Working Group. Reptiles, amphibians, and human *Salmonella* infection: a population-based, case-control study. Clin Infect Dis. 2004;38 Suppl 3:S253-61. doi: 10.1086/381594.

163. Cummings KJ, Warnick LD, Davis MA, Eckmann K, Grohn YT, Hoelzer K, MacDonald K, Root TP, Siler JD, McGuire SM, Wiedmann M, Wright EM, Zansky SM, Besser TE. Farm animal contact as risk factor for transmission of bovine-associated *Salmonella* subtypes. Emerg Infect Dis. 2012;18(12):1929-36. doi: 10.3201/eid1812.110831.

164. Younus M, Wilkins MJ, Davies HD, Rahbar MH, Funk J, Nguyen C, Siddiqi AE, Cho S, Saeed AM. The role of exposures to animals and other risk factors in sporadic, non-typhoidal *Salmonella* infections in Michigan children. Zoonoses Public Health. 2010;57(7-8):e170-6. doi: 10.1111/j.1863-2378.2010.01324.x.

165. Doorduyn Y, Van Den Brandhof WE, Van Duynhoven YT, Wannet WJ, Van Pelt W. Risk factors for *Salmonella* Enteritidis and Typhimurium (DT104 and non-DT104) infections in The Netherlands: predominant roles for raw eggs in Enteritidis and sandboxes in Typhimurium infections. Epidemiol Infect. 2006;134(3):617-26. doi: 10.1017/S0950268805005406.

166. Cavallaro E, Date K, Medus C, Meyer S, Miller B, Kim C, Nowicki S, Cosgrove S, Sweat D, Phan Q, Flint J, Daly ER, Adams J, Hyytia-Trees E, Gerner-Smidt P, Hoekstra RM, Schwensohn C, Langer A, Sodha SV, Rogers MC, Angulo FJ, Tauxe RV, Williams IT, Behravesh CB for the Salmonella Typhimurium Outbreak Investigation Team. *Salmonella* Typhimurium infections associated with peanut products. N Engl J Med. 2011;365(7):601-10. doi: 10.1056/NEJMoa1011208.

167. Bayer C, Bernard H, Prager R, Rabsch W, Hiller P, Malorny B, Pfefferkorn B, Frank C, de Jong A, Friesema I, Stark K, Rosner B. An outbreak of *Salmonella* Newport associated with mung bean sprouts in Germany and the Netherlands, October to November 2011. Euro Surveill. 2014;19(1).

168. Thamlikitkul V, Dhiraputra C, Paisarnsinsup T, Chareandee C. Non-typhoidal *Salmonella* bacteraemia: clinical features and risk factors. Trop Med Int Health. 1996;1(4):443-8.

169. Uche IV, MacLennan CA, Saul A. A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal *Salmonella* (iNTS) Disease in Africa (1966 to 2014). PLoS Negl Trop Dis. 2017;11(1):e0005118. doi: 10.1371/journal.pntd.0005118.

170. Switaj TL, Winter KJ, Christensen SR. Diagnosis and Management of Foodborne Illness. Am Fam Physician. 2015;92(5):358-65.

171. Mody RK, Griffin PM. Editorial Commentary: Increasing Evidence That Certain Antibiotics Should Be Avoided for Shiga Toxin-Producing *Escherichia coli* Infections: More Data Needed. Clin Infect Dis. 2016;62(10):1259-61. doi: 10.1093/cid/ciw101.

172. Freedman SB, Xie J, Neufeld MS, Hamilton WL, Hartling L, Tarr PI, Alberta Provincial Pediatric Enteric Infection T, Nettel-Aguirre A, Chuck A, Lee B, Johnson D, Currie G, Talbot J, Jiang J, Dickinson J, Kellner J, MacDonald J, Svenson L, Chui L, Louie M, Lavoie M, Eltorki M, Vanderkooi O, Tellier R, Ali S, Drews S, Graham T, Pang XL. Shiga Toxin-Producing *Escherichia coli* Infection, Antibiotics, and Risk of Developing Hemolytic Uremic Syndrome: A Meta-analysis. Clin Infect Dis. 2016;62(10):1251-8. doi: 10.1093/cid/ciw099.

173. Ikeda K, Ida O, Kimoto K, Takatorige T, Nakanishi N, Tatara K. Effect of early fosfomycin treatment on prevention of hemolytic uremic syndrome accompanying *Escherichia coli* O157:H7 infection. Clin Nephrol. 1999;52(6):357-62.

174. Zhang X, McDaniel AD, Wolf LE, Keusch GT, Waldor MK, Acheson DW. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J Infect Dis. 2000;181(2):664-70. doi: 10.1086/315239.

175. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N Engl J Med. 2000;342(26):1930-6. doi: 10.1056/NEJM200006293422601.

176. Waldor MK, Friedman DI. Phage regulatory circuits and virulence gene expression. Curr Opin Microbiol. 2005;8(4):459-65. doi: 10.1016/j.mib.2005.06.001.

177. Walterspiel JN, Ashkenazi S, Morrow AL, Cleary TG. Effect of subinhibitory concentrations of antibiotics on extracellular Shiga-like toxin I. Infection. 1992;20(1):25-9.

178. McGannon CM, Fuller CA, Weiss AA. Different classes of antibiotics differentially influence shiga toxin production. Antimicrob Agents Chemother. 2010;54(9):3790-8. doi: 10.1128/AAC.01783-09.

179. Smith KE, Wilker PR, Reiter PL, Hedican EB, Bender JB, Hedberg CW. Antibiotic Treatment of *Escherichia coli* O157 Infection and the Risk of Hemolytic Uremic Syndrome, Minnesota. Pediatr Infect Dis J. 2012;31(1):37-41. doi: 10.1097/INF.0b013e31823096a8.

180. Nelson JM, Griffin PM, Jones TF, Smith KE, Scallan E. Antimicrobial and antimotility agent use in persons with shiga toxin-producing *Escherichia coli* O157 infection in foodnet sites. Clin Infect Dis. 2011;52(9):1130-2.

181. Dryden MS, Gabb RJ, Wright SK. Empirical treatment of severe acute community-acquired gastroenteritis with ciprofloxacin. Clin Infect Dis. 1996;22(6):1019-25.

182. Sanchez-Vargas FM, Abu-El-Haija MA, Gomez-Duarte OG. *Salmonella* infections: an update on epidemiology, management, and prevention. Travel Med Infect Dis. 2011;9(6):263-77. doi: 10.1016/j.tmaid.2011.11.001.

183. Burkhardt JE, Walterspiel JN, Schaad UB. Quinolone arthropathy in animals versus children. Clin Infect Dis. 1997;25(5):1196-204.

184. Choi S-H, Kim EY, Kim Y-J. Systemic use of fluoroquinolone in children. Korean Journal of Pediatrics. 2013;56(5):196-201. doi: 10.3345/kjp.2013.56.5.196.

185. Patel K, Goldman JL. Safety Concerns Surrounding Quinolone Use in Children. J Clin Pharmacol. 2016;56(9):1060-75. doi: 10.1002/jcph.715.

186. Karande SC, Kshirsagar NA. Adverse drug reaction monitoring of ciprofloxacin in pediatric practice. Indian Pediatr. 1992;29(2):181-8.

187. Lightfoot NF, Ahmad F, Cowden J. Management of institutional outbreaks of *Salmonella* gastroenteritis. J Antimicrob Chemother. 1990;26 Suppl F:37-46.

188. Onwuezobe IA, Oshun PO, Odigwe CC. Antimicrobials for treating symptomatic non-typhoidal *Salmonella* infection. Cochrane Database Syst Rev. 2012;11:CD001167. doi: 10.1002/14651858.CD001167.pub2.

189. Aserkoff B, Bennett JV. Effect of Antibiotic Therapy in Acute Salmonellosis on the Fecal Excretion of Salmonellae. N Engl J Med. 1969;281(12):636-40. doi: 10.1056/nejm196909182811202.

190. Sirinavin S, Garner P. Antibiotics for treating salmonella gut infections. Cochrane Database Syst Rev. 2000(2):CD001167. doi: 10.1002/14651858.CD001167.

191. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

192. Levy SB, Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med. 2004;10(12 Suppl):S122-9. doi: 10.1038/nm1145.

193. Holmberg SD, Solomon SL, Blake PA. Health and economic impacts of antimicrobial resistance. Rev Infect Dis. 1987;9(6):1065-78.

194. O'Neill J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. Review on Antimicrobial Resistance. 2014:1-16.

195. Clinical & Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

196. Centers for Disease Control and Prevention. Data from: National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC. 2018.

197. National Antimicrobial Resistance Monitoring System. NARMS 2011 Executive Report. 2011.

198. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. 2018.

199. European Centre for Disease Prevention and Control. European Antimicrobial Resistance Surveillance Network (EARS-Net). 2018.

200. European Center for Disease Prevention and Control. Surveillance of Antimicrobial Resistance in Europe. 2016.

201. World Health Organization. Global Antimicrobial Resistance Surveillance System (GLASS)2018.

202. World Health Organization. Global antimicrobial resistance surveillance system (GLASS) report2018.

203. Kim HH, Samadpour M, Grimm L, Clausen CR, Besser TE, Baylor M, Kobayashi JM, Neill MA, Schoenknecht FD, Tarr PI. Characteristics of antibiotic-resistant *Escherichia coli* O157:H7 in Washington State, 1984-1991. J Infect Dis. 1994;170(6):1606-9.

204. Schroeder CM, White DG, Ge B, Zhang Y, McDermott PF, Ayers S, Zhao S, Meng J. Isolation of antimicrobial-resistant *Escherichia coli* from retail meats purchased in Greater Washington, DC, USA. Int J Food Microbiol. 2003;85(1-2):197-202.

205. Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD, Meng J. Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Appl Environ Microbiol. 2002;68(2):576-81.

206. Meng J, Zhao S, Doyle MP, Joseph SW. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J Food Prot. 1998;61(11):1511-4.

207. Khan A, Das SC, Ramamurthy T, Sikdar A, Khanam J, Yamasaki S, Takeda Y, Nair GB. Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin-producing *Escherichia coli* isolates from diverse sources in Calcutta, India. J Clin Microbiol. 2002;40(6):2009-15.

208. Canizalez-Roman A, Gonzalez-Nunez E, Vidal JE, Flores-Villasenor H, Leon-Sicairos N. Prevalence and antibiotic resistance profiles of diarrheagenic *Escherichia coli* strains isolated from food items in northwestern Mexico. Int J Food Microbiol. 2013;164(1):36-45. doi: 10.1016/j.ijfoodmicro.2013.03.020.

209. Mahanti A, Samanta I, Bandopaddhay S, Joardar SN, Dutta TK, Batabyal S, Sar TK, Isore DP. Isolation, molecular characterization and antibiotic resistance of Shiga Toxin-Producing *Escherichia coli* (STEC) from buffalo in India. Lett Appl Microbiol. 2013;56(4):291-8. doi: 10.1111/lam.12048.

210. Oldfield EC, Wallace MR. The Role of Antibiotics in the Treatment of Infectious Diarrhea. Gastroenterol Clin North Am. 2001;30(3):817-35. doi: https://doi.org/10.1016/S0889-8553(05)70212-0.

211. Diniz-Santos DR, Silva LR, Silva N. Antibiotics for the empirical treatment of acute infectious diarrhea in children. Braz J Infect Dis. 2006;10:217-27.

212. Stephan R, Schumacher S. Resistance patterns of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from animals, food and asymptomatic human carriers in Switzerland. Lett Appl Microbiol. 2001;32(2):114-7. doi: doi:10.1046/j.1472-765x.2001.00867.x.

213. Schroeder CM, Meng J, Zhao S, DebRoy C, Torcolini J, Zhao C, McDermott PF, Wagner DD, Walker RD, White DG. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerg Infect Dis. 2002;8(12):1409-14. doi: 10.3201/eid0812.020070.

214. Mukherjee S, Mosci RE, Anderson CM, Snyder BA, Collins J, Rudrik JT, Manning SD. Antimicrobial Drug-Resistant Shiga Toxin-Producing *Escherichia coli* Infections, Michigan, USA. Emerg Infect Dis. 2017;23(9):1609-11. doi: 10.3201/eid2309.170523.

215. Mora A, Blanco JE, Blanco M, Alonso MP, Dhabi G, Echeita A, Gonzalez EA, Bernardez MI, Blanco J. Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. Res Microbiol. 2005;156(7):793-806. doi: 10.1016/j.resmic.2005.03.006.

216. Fukushima H, Hoshina K, Gomyoda M. Long-term survival of shiga toxin-producing *Escherichia coli* O26, O111, and O157 in bovine feces. Appl Environ Microbiol. 1999;65(11):5177-81.

217. Bolton DJ, Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell DA. Incidence and survival of non-O157 verocytotoxigenic *Escherichia coli* in soil. Journal of Applied Microbiology. 2011;111(2):484-90. doi: doi:10.1111/j.1365-2672.2011.05057.x.

218. Martínez JL. Antibiotics and Antibiotic Resistance Genes in Natural Environments. Science. 2008;321(5887):365.

219. Peng S, Feng Y, Wang Y, Guo X, Chu H, Lin X. Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. J Hazard Mater. 2017;340:16-25. Epub 2017/07/18. doi: 10.1016/j.jhazmat.2017.06.059.

220. Shah DH, Paul NC, Sischo WC, Crespo R, Guard J. Population dynamics and antimicrobial resistance of the most prevalent poultry-associated *Salmonella* serotypes. Poult Sci. 2017;96(3):687-702. doi: 10.3382/ps/pew342.

221. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

222. Medalla F, Gu W, Mahon BE, Judd M, Folster J, Griffin PM, Hoekstra RM. Estimated Incidence of Antimicrobial Drug-Resistant Nontyphoidal *Salmonella* Infections, United States, 2004-2012. Emerg Infect Dis. 2016;23(1):29-37. doi: 10.3201/eid2301.160771.

223. Soler P, González-Sanz R, Bleda MJ, Hernández G, Echeíta A, Usera MA. Antimicrobial resistance in non-typhoidal *Salmonella* from human sources, Spain, 2001–2003. J Antimicrob Chemother. 2006;58(2):310-4. doi: 10.1093/jac/dkl223.

224. Hoge CW, Gambel JM, Srijan A, Pitarangsi C, Echeverria P. Trends in Antibiotic Resistance Among Diarrheal Pathogens Isolated in Thailand Over 15 Years. Clin Infect Dis. 1998;26(2):341-5. doi: 10.1086/516303.

225. Burke L, Hopkins KL, Meunier D, de Pinna E, Fitzgerald-Hughes D, Humphreys H, Woodford N. Resistance to third-generation cephalosporins in human non-typhoidal *Salmonella enterica* isolates from England and Wales, 2010-12. J Antimicrob Chemother. 2014;69(4):977-81. doi: 10.1093/jac/dkt469.

226. Liakopoulos A, Geurts Y, Dierikx CM, Brouwer MSM, Kant A, Wit B, Heymans R, van Pelt W, Mevius DJ. Extended-Spectrum Cephalosporin-Resistant *Salmonella enterica* serovar Heidelberg Strains, the Netherlands. Emerg Infect Dis. 2016;22(7):1257-61. doi: 10.3201/eid2207.151377.

227. Lay KS, Vuthy Y, Song P, Phol K, Sarthou JL. Prevalence, numbers and antimicrobial susceptibilities of *Salmonella* serovars and *Campylobacter* spp. in retail poultry in Phnom Penh, Cambodia. J Vet Med Sci. 2011;73(3):325-9.

228. Boonmar S, Markvichitr K, Chaunchom S, Chanda C, Bangtrakulnonth A, Pornrunangwong S, Yamamoto S, Suzuki D, Kozawa K, Kimura H, Morita Y. *Salmonella* prevalence in slaughtered buffaloes and pigs and antimicrobial susceptibility of isolates in Vientiane, Lao People's Democratic Republic. J Vet Med Sci. 2008;70(12):1345-8.

229. National Antimicrobial Resistance Monitoring System. NARMS: 2010 Executive Report. 2010.

230. Emborg HD, Baggesen DL, Aarestrup FM. Ten years of antimicrobial susceptibility testing of *Salmonella* from Danish pig farms. J Antimicrob Chemother. 2008;62(2):360-3. doi: 10.1093/jac/dkn196.

231. Gebreyes WA, Altier C. Molecular characterization of multidrug-resistant *Salmonella* enterica subsp. enterica serovar Typhimurium isolates from swine. J Clin Microbiol. 2002;40(8):2813-22.

232. Akullian A, Montgomery JM, John-Stewart G, Miller SI, Hayden HS, Radey MC, Hager KR, Verani JR, Ochieng JB, Juma J, Katieno J, Fields B, Bigogo G, Audi A, Walson J. Multi-drug resistant non-typhoidal *Salmonella* associated with invasive disease in western Kenya. PLoS Negl Trop Dis. 2018;12(1):e0006156. doi: 10.1371/journal.pntd.0006156.

233. Parisi A, Crump JA, Glass K, Howden BP, Furuya-Kanamori L, Vilkins S, Gray DJ, Kirk MD. Health Outcomes from Multidrug-Resistant *Salmonella* Infections in High-Income Countries: A Systematic Review and Meta-Analysis. Foodborne Pathog Dis. 2018;15(7):428-36. doi: 10.1089/fpd.2017.2403.

234. Varma JK, Molbak K, Barrett TJ, Beebe JL, Jones TF, Rabatsky-Ehr T, Smith KE, Vugia DJ, Chang HG, Angulo FJ. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J Infect Dis. 2005;191(4):554-61. doi: 10.1086/427263.

235. Barlow RS, Debess EE, Winthrop KL, Lapidus JA, Vega R, Cieslak PR. Travel-associated antimicrobial drug-resistant nontyphoidal Salmonellae, 2004-2009. Emerg Infect Dis. 2014;20(4):603-11. doi: 10.3201/eid2004.131063.

236. Zerr DM, Miles-Jay A, Kronman MP, Zhou C, Adler AL, Haaland W, Weissman SJ, Elward A, Newland JG, Zaoutis T, Qin X. Previous Antibiotic Exposure Increases Risk of Infection with Extended-Spectrum-beta-Lactamase- and AmpC-Producing *Escherichia coli* and *Klebsiella pneumoniae* in Pediatric Patients. Antimicrob Agents Chemother. 2016;60(7):4237-43. doi: 10.1128/AAC.00187-16.

237. Hillier S, Roberts Z, Dunstan F, Butler C, Howard A, Palmer S. Prior antibiotics and risk of antibiotic-resistant community-acquired urinary tract infection: a case-control study. J Antimicrob Chemother. 2007;60(1):92-9. doi: 10.1093/jac/dkm141.

238. Augustin P, Kermarrec N, Muller-Serieys C, Lasocki S, Chosidow D, Marmuse J-P, Valin N, Desmonts J-M, Montravers P. Risk factors for multidrug resistant bacteria and optimization of empirical antibiotic therapy in postoperative peritonitis. Critical Care. 2010;14(1):R20. doi: 10.1186/cc8877.

239. Sonmezer MC, Ertem G, Erdinc FS, Kaya Kilic E, Tulek N, Adiloglu A, Hatipoglu C. Evaluation of Risk Factors for Antibiotic Resistance in Patients with Nosocomial Infections Caused by *Pseudomonas aeruginosa*. Can J Infect Dis Med Microbiol. 2016;2016:1321487. doi: 10.1155/2016/1321487.

240. Allen UD, MacDonald N, Fuite L, Chan F, Stephens D. Risk factors for resistance to "first-line" antimicrobials among urinary tract isolates of *Escherichia coli* in children. CMAJ. 1999;160(10):1436-40.

241. Steinke DT, Seaton RA, Phillips G, MacDonald TM, Davey PG. Factors associated with trimethoprim-resistant bacteria isolated from urine samples. J Antimicrob Chemother. 1999;43(6):841-3.

242. Sotto A, De Boever CM, Fabbro-Peray P, Gouby A, Sirot D, Jourdan J. Risk factors for antibiotic-resistant *Escherichia coli* isolated from hospitalized patients with urinary tract infections: a prospective study. J Clin Microbiol. 2001;39(2):438-44. doi: 10.1128/JCM.39.2.438-444.2001.

243. Ryan CA, Nickels MK, Hargrett-Bean NT, et al. Massive outbreak of antimicrobial-resistant salmonellosis traced to pasteurized milk. JAMA. 1987;258(22):3269-74. doi: 10.1001/jama.1987.03400220069039.

244. Seidman JC, Anitha KP, Kanungo R, Bourgeois AL, Coles CL. Risk factors for antibiotic-resistant *E. coli* in children in a rural area. Epidemiol Infect. 2009;137(6):879-88. doi: 10.1017/S0950268808001519.

245. Fornasini M, Reves RR, Murray BE, Morrow AL, Pickering LK. Trimethoprim-Resistant *Escherichia coli* in Households of Children Attending Day Care Centers. J Infect Dis. 1992;166(2):326-30.

246. Sattler CA, Mason EO, Jr., Kaplan SL. Prospective comparison of risk factors and demographic and clinical characteristics of community-acquired, methicillin-resistant versus methicillin-susceptible *Staphylococcus aureus* infection in children. Pediatr Infect Dis J. 2002;21(10):910-7. doi: 10.1097/01.inf.0000034268.59915.6f.

247. Flamm RK, Weaver MK, Thornsberry C, Jones ME, Karlowsky JA, Sahm DF. Factors associated with relative rates of antibiotic resistance in *Pseudomonas aeruginosa* isolates

tested in clinical laboratories in the United States from 1999 to 2002. Antimicrob Agents Chemother. 2004;48(7):2431-6. doi: 10.1128/AAC.48.7.2431-2436.2004.

248. Alos JI, Serrano MG, Gomez-Garces JL, Perianes J. Antibiotic resistance of *Escherichia coli* from community-acquired urinary tract infections in relation to demographic and clinical data. Clin Microbiol Infect. 2005;11(3):199-203. doi: 10.1111/j.1469-0691.2004.01057.x.

249. Liang Z, Ke B, Deng X, Liang J, Ran L, Lu L, He D, Huang Q, Ke C, Li Z, Yu H, Klena JD, Wu S. Serotypes, seasonal trends, and antibiotic resistance of non-typhoidal *Salmonella* from human patients in Guangdong Province, China, 2009-2012. BMC Infect Dis. 2015;15:53. doi: 10.1186/s12879-015-0784-4.

250. Shorr AF, Zilberberg MD, Micek ST, Kollef MH. Prediction of infection due to antibiotic-resistant bacteria by select risk factors for health care-associated pneumonia. Arch Intern Med. 2008;168(20):2205-10. doi: 10.1001/archinte.168.20.2205.

251. Cha W, Mosci R, Wengert SL, Singh P, Newton DW, Salimnia H, Lephart P, Khalife W, Mansfield LS, Rudrik JT, Manning SD. Antimicrobial Susceptibility Profiles of Human *Campylobacter jejuni* Isolates and Association with Phylogenetic Lineages. Front Microbiol. 2016;7:589. doi: 10.3389/fmicb.2016.00589.

252. Kassenborg HD, Smith KE, Vugia DJ, Rabatsky-Ehr T, Bates MR, Carter MA, Dumas NB, Cassidy MP, Marano N, Tauxe RV, Angulo FJ, for the Emerging Infections Program FoodNet Working Group. Fluoroquinolone-Resistant *Campylobacter* Infections: Eating Poultry Outside of the Home and Foreign Travel Are Risk Factors. Clin Infect Dis. 2004;38(Supplement_3):S279-S84. doi: 10.1086/381597.

253. Sahoo KC, Tamhankar AJ, Sahoo S, Sahu PS, Klintz SR, Lundborg CS. Geographical Variation in Antibiotic-Resistant *Escherichia coli* Isolates from Stool, Cow-Dung and Drinking Water. Int J Environ Res Public Health. 2012;9(3):746-59. doi: 10.3390/ijerph9030746.

254. Goossens H, Ferech M, Vander Stichele R, Elseviers M. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. The Lancet. 2005;365(9459):579-87. doi: https://doi.org/10.1016/S0140-6736(05)17907-0.

255. Gould IM. A review of the role of antibiotic policies in the control of antibiotic resistance. J Antimicrob Chemother. 1999;43(4):459-65. doi: 10.1093/jac/43.4.459.

256. Seppala H, Klaukka T, Vuopio-Varkila J, Muotiala A, Helenius H, Lager K, Huovinen P. The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. Finnish Study Group for Antimicrobial Resistance. N Engl J Med. 1997;337(7):441-6. doi: 10.1056/nejm199708143370701.

257. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, Pfyffer GE, Borrell S, Dubuis O, Bruderer T, Siegrist HH, Furrer H, Calmy A, Fehr J, Stalder JM, Ninet B, Bottger EC, Gagneux S, Swiss HIVCS for the Swiss Molecular Epidemiology of Tuberculosis Study Group. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2012;56(6):3047-53. doi: 10.1128/AAC.06460-11.

258. Qumar S, Majid M, Kumar N, Tiwari SK, Semmler T, Devi S, Baddam R, Hussain A, Shaik S, Ahmed N. Genome Dynamics and Molecular Infection Epidemiology of Multidrug-Resistant *Helicobacter pullorum* Isolates Obtained from Broiler and Free-Range Chickens in India. Appl Environ Microbiol. 2017;83(1). doi: 10.1128/AEM.02305-16.

259. Mariam SH, Werngren J, Aronsson J, Hoffner S, Andersson DI. Dynamics of antibiotic resistant *Mycobacterium tuberculosis* during long-term infection and antibiotic treatment. PLoS One. 2011;6(6):e21147. doi: 10.1371/journal.pone.0021147.

260. Donaldson SC, Straley BA, Hegde NV, Sawant AA, DebRoy C, Jayarao BM. Molecular epidemiology of ceftiofur-resistant *Escherichia coli* isolates from dairy calves. Appl Environ Microbiol. 2006;72(6):3940-8. doi: 10.1128/AEM.02770-05.

261. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16(2):161-8. doi: 10.1016/S1473-3099(15)00424-7.

262. Wyrsch ER, Roy Chowdhury P, Chapman TA, Charles IG, Hammond JM, Djordjevic SP. Genomic Microbial Epidemiology Is Needed to Comprehend the Global Problem of Antibiotic Resistance and to Improve Pathogen Diagnosis. Front Microbiol. 2016;7:843. doi: 10.3389/fmicb.2016.00843.

263. Klemm E, Dougan G. Advances in Understanding Bacterial Pathogenesis Gained from Whole-Genome Sequencing and Phylogenetics. Cell Host Microbe. 2016;19(5):599-610. doi: 10.1016/j.chom.2016.04.015.

264. Sintchenko V, Holmes EC. The role of pathogen genomics in assessing disease transmission. BMJ. 2015;350:h1314. doi: 10.1136/bmj.h1314.

265. Voss-Rech D, Potter L, Vaz CS, Pereira DI, Sangioni LA, Vargas AC, de Avila Botton S. Antimicrobial Resistance in Nontyphoidal *Salmonella* Isolated from Human and Poultry-Related Samples in Brazil: 20-Year Meta-Analysis. Foodborne Pathog Dis. 2017;14(2):116-24. doi: 10.1089/fpd.2016.2228.

266. Perron GG, Bell G, Quessy S. Parallel evolution of multidrug-resistance in *Salmonella enterica* isolated from swine. FEMS Microbiol Lett. 2008;281(1):17-22. doi: 10.1111/j.1574-6968.2007.01045.x.

267. Ratnam S, March SB, Ahmed R, Bezanson GS, Kasatiya S. Characterization of *Escherichia coli* serotype O157:H7. J Clin Microbiol. 1988;26(10):2006-12.

268. Bopp CA, Greene KD, Downes FP, Sowers EG, Wells JG, Wachsmuth IK. Unusual verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis. J Clin Microbiol. 1987;25(8):1486-9.

269. Colello R, Etcheverria AI, Di Conza JA, Gutkind GO, Padola NL. Antibiotic resistance and integrons in Shiga toxin-producing *Escherichia coli* (STEC). Braz J Microbiol. 2015;46(1):1-5. doi: 10.1590/S1517-838246120130698.

270. Askar M, Faber MS, Frank C, Bernard H, Gilsdorf A, Fruth A, Prager R, Hohle M, Suess T, Wadl M, Krause G, Stark K, Werber D. Update on the ongoing outbreak of haemolytic uraemic syndrome due to Shiga toxin-producing *Escherichia coli* (STEC) serotype O104, Germany, May 2011. Euro Surveill. 2011;16(22).

271. Ahmed SA, Awosika J, Baldwin C, Bishop-Lilly KA, Biswas B, Broomall S, Chain PS, Chertkov O, Chokoshvili O, Coyne S, Davenport K, Detter JC, Dorman W, Erkkila TH, Folster JP, Frey KG, George M, Gleasner C, Henry M, Hill KK, Hubbard K, Insalaco J, Johnson S, Kitzmiller A, Krepps M, Lo CC, Luu T, McNew LA, Minogue T, Munk CA, Osborne B, Patel M, Reitenga KG, Rosenzweig CN, Shea A, Shen X, Strockbine N, Tarr C, Teshima H, van Gieson E, Verratti K, Wolcott M, Xie G, Sozhamannan S, Gibbons HS for the Threat Characterization Consortium. Genomic comparison of *Escherichia coli* O104:H4 isolates from 2009 and 2011 reveals plasmid, and prophage heterogeneity, including shiga toxin encoding phage stx2. PLoS One. 2012;7(11):e48228. doi: 10.1371/journal.pone.0048228.

272. Valat C, Haenni M, Saras E, Auvray F, Forest K, Oswald E, Madec JY. CTX-M-15 extended-spectrum beta-lactamase in a shiga toxin-producing *Escherichia coli* isolate of serotype O111:H8. Appl Environ Microbiol. 2012;78(4):1308-9. doi: 10.1128/AEM.06997-11.

273. Ishii Y, Kimura S, Alba J, Shiroto K, Otsuka M, Hashizume N, Tamura K, Yamaguchi K. Extended-spectrum beta-lactamase-producing Shiga toxin gene (Stx1)-positive *Escherichia coli* O26:H11: a new concern. J Clin Microbiol. 2005;43(3):1072-5. doi: 10.1128/JCM.43.3.1072-1075.2005.

274. Zhao S, White DG, Ge B, Ayers S, Friedman S, English L, Wagner D, Gaines S, Meng J. Identification and characterization of integron-mediated antibiotic resistance among Shiga toxin-producing *Escherichia coli* isolates. Appl Environ Microbiol. 2001;67(4):1558-64. doi: 10.1128/AEM.67.4.1558-1564.2001.

275. Maidhof H, Guerra B, Abbas S, Elsheikha HM, Whittam TS, Beutin L. A multiresistant clone of Shiga toxin-producing *Escherichia coli* O118:[H16] is spread in cattle and humans over different European countries. Appl Environ Microbiol. 2002;68(12):5834-42.

276. Adesiji YO, Deekshit VK, Karunasagar I. Antimicrobial-resistant genes associated with *Salmonella* spp. isolated from human, poultry, and seafood sources. Food Sci Nutr. 2014;2(4):436-42. doi: 10.1002/fsn3.119.

277. Sandvang D, Aarestrup FM, Jensen LB. Characterisation of integrons and antibiotic resistance genes in Danish multiresistant *Salmonella enterica* Typhimurium DT104. FEMS Microbiol Lett. 1998;160(1):37-41.

278. Ridley A, Threlfall EJ. Molecular Epidemiology of Antibiotic Resistance Genes in Multiresistant Epidemic *Salmonella* Typhimurium DT 104. Microb Drug Resist. 1998;4(2):113-8. doi: 10.1089/mdr.1998.4.113.

279. Horii T, Kimura T, Sato K, Shibayama K, Ohta M. Emergence of fosfomycin-resistant isolates of Shiga-like toxin-producing *Escherichia coli* O26. Antimicrob Agents Chemother. 1999;43(4):789-93.

280. Almeida F, Seribelli AA, Medeiros MIC, Rodrigues DDP, MelloVarani A, Luo Y, Allard MW, Falcao JP. Phylogenetic and antimicrobial resistance gene analysis of *Salmonella* Typhimurium strains isolated in Brazil by whole genome sequencing. PLoS One. 2018;13(8):e0201882. doi: 10.1371/journal.pone.0201882.

281. Gostev V, Kruglov A, Kalinogorskaya O, Dmitrenko O, Khokhlova O, Yamamoto T, Lobzin Y, Ryabchenko I, Sidorenko S. Molecular epidemiology and antibiotic resistance of methicillin-resistant *Staphylococcus aureus* circulating in the Russian Federation. Infect Genet Evol. 2017;53:189-94. doi: 10.1016/j.meegid.2017.06.006.

282. Kong H, Yu F, Zhang W, Li X, Wang H. Molecular Epidemiology and Antibiotic Resistance Profiles of Methicillin-Resistant *Staphylococcus aureus* Strains in a Tertiary Hospital in China. Front Microbiol. 2017;8:838. doi: 10.3389/fmicb.2017.00838.

283. O'Brien TF, Hopkins JD, Gilleece ES, Medeiros AA, Kent RL, Blackburn BO, Holmes MB, Reardon JP, Vergeront JM, Schell WL, Christenson E, Bissett ML, Morse EV. Molecular epidemiology of antibiotic resistance in *Salmonella* from animals and human beings in the United States. N Engl J Med. 1982;307(1):1-6. doi: 10.1056/NEJM198207013070101.

284. Tassios PT, Markogiannakis A, Vatopoulos AC, Katsanikou E, Velonakis EN, Kourea-Kremastinou J, Legakis NJ. Molecular epidemiology of antibiotic resistance of *Salmonella* Enteritidis during a 7-year period in Greece. J Clin Microbiol. 1997;35(6):1316-21.

285. Centers for Disease Control and Prevention. Pulsed-field Gel Electrophoresis (PFGE). 2018.

286. Belén A, Pavón I, Maiden MCJ. Multilocus Sequence Typing. Methods in molecular biology (Clifton, NJ). 2009;551:129-40. doi: 10.1007/978-1-60327-999-4_11.

287. Isiko J, Khaitsa M, Bergholz TM. Novel sequence types of non-O157 Shiga toxin-producing *Escherichia coli* isolated from cattle. Lett Appl Microbiol. 2015;60(6):552-7. doi: 10.1111/lam.12404.

288. Eichhorn I, Heidemanns K, Semmler T, Kinnemann B, Mellmann A, Harmsen D, Anjum MF, Schmidt H, Fruth A, Valentin-Weigand P, Heesemann J, Suerbaum S, Karch H, Wieler LH. Highly virulent non-O157 enterohemorrhagic *Escherichia coli* (EHEC) serotypes reflect similar phylogenetic lineages, providing new insights into the evolution of EHEC. Appl Environ Microbiol. 2015;81:7041-7.

289. Habing GG, Manning S, Bolin C, Cui Y, Rudrik J, Dietrich S, Kaneene JB. Within-Farm Changes in Dairy Farm-Associated *Salmonella* Subtypes and Comparison to Human Clinical Isolates in Michigan, 2000-2001 and 2009. Appl Environ Microbiol. 2015;81(17):5724-35. doi: 10.1128/AEM.00899-15.

290. Noller AC, Mcellistrem MC, Stine OC, Morris G, Boxrud DJ, Dixon B, Lee H, Morris JG, Harrison LH. Multilocus Sequence Typing Reveals a Lack of Diversity among *Escherichia coli* O157 : H7 Isolates That Are Distinct by Pulsed-Field Gel Electrophoresis. J Clin Microbiol. 2003;41(2):675-9.

291. Manning SD, Motiwala AS, Springman AC, Qi W, Lacher DW, Ouellette LM, Mladonicky JM, Somsel P, Rudrik JT, Dietrich SE, Zhang W, Swaminathan B, Alland D, Whittam TS. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc Natl Acad Sci U S A. 2008;105(12):4868-73. Epub 2008/03/12. doi: 10.1073/pnas.0710834105.

292. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66(10):4555-8.

293. Herzer PJ, Inouye S, Inouye M, Whittam TS. Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of *Escherichia coli*. J Bacteriol. 1990;172(11):6175-81.

294. Carlos C, Pires MM, Stoppe NC, Hachich EM, Sato MI, Gomes TA, Amaral LA, Ottoboni LM. *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. BMC Microbiol. 2010;10:161. doi: 10.1186/1471-2180-10-161.

295. Stoppe NC, Silva JS, Carlos C, Sato MIZ, Saraiva AM, Ottoboni LMM, Torres TT. Worldwide Phylogenetic Group Patterns of *Escherichia coli* from Commensal Human

and Wastewater Treatment Plant Isolates. Front Microbiol. 2017;8:2512. doi: 10.3389/fmicb.2017.02512.

296. Chakraborty A, Saralaya V, Adhikari P, Shenoy S, Baliga S, Hegde A. Characterization of *Escherichia coli* Phylogenetic Groups Associated with Extraintestinal Infections in South Indian Population. Ann Med Health Sci Res. 2015;5(4):241-6. doi: 10.4103/2141-9248.160192.

297. Moreno E, Prats G, Sabate M, Perez T, Johnson JR, Andreu A. Quinolone, fluoroquinolone and trimethoprim/sulfamethoxazole resistance in relation to virulence determinants and phylogenetic background among uropathogenic *Escherichia coli*. J Antimicrob Chemother. 2006;57(2):204-11. doi: 10.1093/jac/dki468.

298. Johnson JR, Kuskowski MA, Owens K, Gajewski A, Winokur PL. Phylogenetic origin and virulence genotype in relation to resistance to fluoroquinolones and/or extended-spectrum cephalosporins and cephamycins among *Escherichia coli* isolates from animals and humans. J Infect Dis. 2003;188(5):759-68. doi: 10.1086/377455.

299. Giufre M, Graziani C, Accogli M, Luzzi I, Busani L, Cerquetti M for the *Escherichia coli* Study Group. *Escherichia coli* of human and avian origin: detection of clonal groups associated with fluoroquinolone and multidrug resistance in Italy. J Antimicrob Chemother. 2012;67(4):860-7. doi: 10.1093/jac/dkr565.

300. Akhtardanesh B, Ghanbarpour R, Ganjalikhani S, Gazanfari P. Determination of antibiotic resistance genes in relation to phylogenetic background in *Escherichia coli* isolates from fecal samples of healthy pet cats in Kerman city. Vet Res Forum. 2016;7(4):301-8.

301. Ferdous M, Kooistra-Smid AM, Zhou K, Rossen JW, Friedrich AW. Virulence, Antimicrobial Resistance Properties and Phylogenetic Background of Non-H7 Enteropathogenic *Escherichia coli* O157. Front Microbiol. 2016;7:1540. doi: 10.3389/fmicb.2016.01540.

302. Johnson JR, Johnston B, Clabots C, Kuskowski MA, Pendyala S, Debroy C, Nowicki B, Rice J. *Escherichia coli* sequence type ST131 as an emerging fluoroquinolone-resistant uropathogen among renal transplant recipients. Antimicrob Agents Chemother. 2010;54(1):546-50. doi: 10.1128/AAC.01089-09.

303. Petty NK, Ben Zakour NL, Stanton-Cook M, Skippington E, Totsika M, Forde BM, Phan MD, Gomes Moriel D, Peters KM, Davies M, Rogers BA, Dougan G, Rodriguez-Bano J, Pascual A, Pitout JD, Upton M, Paterson DL, Walsh TR, Schembri MA, Beatson SA. Global dissemination of a multidrug resistant *Escherichia coli* clone. Proc Natl Acad Sci U S A. 2014;111(15):5694-9. doi: 10.1073/pnas.1322678111.

304. Mosquito S, Pons MJ, Riveros M, Ruiz J, Ochoa TJ. Diarrheagenic *Escherichia coli* phylogroups are associated with antibiotic resistance and duration of diarrheal episode. ScientificWorldJournal. 2015;2015:610403. doi: 10.1155/2015/610403.

305. Ingle DJ, Levine MM, Kotloff KL, Holt KE, Robins-Browne RM. Dynamics of antimicrobial resistance in intestinal *Escherichia coli* from children in community settings in South Asia and sub-Saharan Africa. Nat Microbiol. 2018;3(9):1063-73. doi: 10.1038/s41564-018-0217-4.

306. Dallman TJ, Ashton PM, Byrne L, Perry NT, Petrovska L, Ellis R, Allison L, Hanson M, Holmes A, Gunn GJ, Chase-Topping ME, Woolhouse ME, Grant KA, Gally DL, Wain J, Jenkins C. Applying phylogenomics to understand the emergence of Shiga-toxin-producing *Escherichia coli* O157:H7 strains causing severe human disease in the UK. Microb Genom. 2015;1(3):e000029. doi: 10.1099/mgen.0.000029.

307. Liu F, Kariyawasam S, Jayarao BM, Barrangou R, Gerner-Smidt P, Ribot EM, Knabel SJ, Dudley EG. Subtyping *Salmonella enterica* serovar enteritidis isolates from different sources by using sequence typing based on virulence genes and clustered regularly interspaced short palindromic repeats (CRISPRs). Appl Environ Microbiol. 2011;77(13):4520-6. Epub 2011/05/17. doi: 10.1128/aem.00468-11.

308. DiMarzio M, Shariat N, Kariyawasam S, Barrangou R, Dudley EG. Antibiotic Resistance in *Salmonella enterica* Serovar Typhimurium Associates with CRISPR Sequence Type. Antimicrob Agents Chemother. 2013;57(9):4282-9. doi: 10.1128/AAC.00913-13.

309. Alcaine SD, Sukhnanand SS, Warnick LD, Su WL, McGann P, McDonough P, Wiedmann M. Ceftiofur-resistant *Salmonella* strains isolated from dairy farms represent multiple widely distributed subtypes that evolved by independent horizontal gene transfer. Antimicrob Agents Chemother. 2005;49(10):4061-7. doi: 10.1128/AAC.49.10.4061-4067.2005.

310. Izumiya H, Terajima J, Matsushita S, Tamura K, Watanabe H. Characterization of multidrug-resistant *Salmonella enterica* serovar Typhimurium isolated in Japan. J Clin Microbiol. 2001;39(7):2700.

311. Vatopoulos AC, Mainas E, Balis E, Threlfall EJ, Kanelopoulou M, Kalapothalki V, Malamou-Lada H, Legakis NJ. Molecular epidemiology of ampicillin-resistant clinical isolates of *Salmonella* Enteritidis. J Clin Microbiol. 1994;32(5):1322.

312. Yuen CM, Kurbatova EV, Click ES, Cavanaugh JS, Cegielski JP. Association between *Mycobacterium tuberculosis* Complex Phylogenetic Lineage and Acquired Drug Resistance. PLOS ONE. 2013;8(12):e83006. doi: 10.1371/journal.pone.0083006.

313. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. Clin Infect Dis. 2002;34 s93-s106.

314. Schwarz S, Kehrenberg C, Walsh TR. Use of antimicrobial agents in veterinary medicine and food animal production. Int J Antimicrob Agents. 2001;17(6):431-7.

315. US Food and Drug Administration . 2016 annual summary report of sales and distribution data for antimicrobial drugs intended for use in food-producing animals. 2017.

316. Public Health Agency of Canada. Canadian Antimicrobial Resistance Surveillance System 2017 Report. 2017.

317. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, Teillant A, Laxminarayan R. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci. 2015;112(18):5649.

318. Call D, Matthews L, Subbiah M, Liu J. Do antibiotic residues in soils play a role in amplification and transmission of antibiotic resistant bacteria in cattle populations? Front Microbiol. 2013;4(193). doi: 10.3389/fmicb.2013.00193.

319. Smith DL, Harris AD, Johnson JA, Silbergeld EK, Morris JG. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Proc Natl Acad Sci. 2002;99(9):6434.

320. Looft T, Johnson TA, Allen HK, Bayles DO, Alt DP, Stedtfeld RD, Sul WJ, Stedtfeld TM, Chai B, Cole JR, Hashsham SA, Tiedje JM, Stanton TB. In-feed antibiotic effects on the swine intestinal microbiome. Proc Natl Acad Sci U S A. 2012;109(5):1691-6. doi: 10.1073/pnas.1120238109.

321. Starr MP, Reynolds DM. Streptomycin resistance of coliform bacteria from turkeys fed streptomycin. Am J Public Health Nations Health. 1951;41(11 Pt 1):1375-80.

322. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother. 2014;69(3):827-34. doi: 10.1093/jac/dkt443.

323. Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F. Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark. Antimicrob Agents Chemother. 2001;45(7):2054-9. doi: 10.1128/aac.45.7.2054-2059.2001.

324. Ghosh S, LaPara TM. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. 2007;1:191. doi: 10.1038/ismej.2007.31.

325. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP. Quinolone resistance in *Campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother. 1991;27(2):199-208.

326. Fein D, Burton G, Tsutakawa R, Blenden D. Matching of antibiotic resistance patterns of *Escherichia coli* of farm families and their animals. J Infect Dis. 1974;130:274-9.

327. Bates J, Jordens JZ, Griffiths DT. Farm animals as a putative reservoir for vancomycin-resistant enterococcal infection in man. J Antimicrob Chemother. 1994;34(4):507-14. Epub 1994/10/01.

328. Reynaga E, Navarro M, Vilamala A, Roure P, Quintana M, Garcia-Nunez M, Figueras R, Torres C, Lucchetti G, Sabria M. Prevalence of colonization by methicillin-resistant *Staphylococcus aureus* ST398 in pigs and pig farm workers in an area of Catalonia, Spain. BMC Infect Dis. 2016;16(1):716. doi: 10.1186/s12879-016-2050-9.

329. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner DD, Meng J. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med. 2001;345(16):1147-54. doi: 10.1056/NEJMoa010315.

330. Dallal MMS, Doyle MP, Rezadehbashi M, Dabiri H, Sanaei M, Modarresi S, Bakhtiari R, Sharifiy K, Taremi M, Zali MR, Sharifi-Yazdi MK. Prevalence and antimicrobial resistance profiles of *Salmonella* serotypes, *Campylobacter* and *Yersinia* spp. isolated from retail chicken and beef, Tehran, Iran. Food Control. 2010;21(4):388-92. doi: https://doi.org/10.1016/j.foodcont.2009.06.001.

331. Little CL, Richardson JF, Owen RJ, de Pinna E, Threlfall EJ. *Campylobacter* and *Salmonella* in raw red meats in the United Kingdom: Prevalence, characterization and antimicrobial resistance pattern, 2003–2005. Food Microbiol. 2008;25(3):538-43. doi: https://doi.org/10.1016/j.fm.2008.01.001.

332. Hong J, Kim JM, Jung WK, Kim SH, Bae W, Koo HC, Gil J, Kim M, Ser J, Park YH. Prevalence and antibiotic resistance of *Campylobacter* spp. isolated from chicken meat, pork, and beef in Korea, from 2001 to 2006. J Food Prot. 2007;70(4):860-6.

333. Cui S, Ge B, Zheng J, Meng J. Prevalence and antimicrobial resistance of *Campylobacter* spp. and *Salmonella* serovars in organic chickens from Maryland retail stores. Appl Environ Microbiol. 2005;71(7):4108-11. doi: 10.1128/AEM.71.7.4108-4111.2005.

334. Amézquita-López BA, Quiñones B, Soto-Beltrán M, Lee BG, Yambao JC, Lugo-Melchor OY, Chaidez C. Antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* O157 and Non-O157 recovered from domestic farm animals in rural communities in Northwestern Mexico. Antimicrob Resist Infect Control. 2016;5:1. doi: 10.1186/s13756-015-0100-5.

335. Srinivasan V, Nguyen LT, Headrick SI, Murinda SE, Oliver SP. Antimicrobial resistance patterns of Shiga toxin-producing *Escherichia coli* O157:H7 and O157:H7- from different origins. Microb Drug Resist. 2007;13(1):44-51. doi: 10.1089/mdr.2006.9996.

336. Adamu MS, Ugochukwu ICI, Idoko SI, Kwabugge YA, Abubakar NS, Ameh JA. Virulent gene profile and antibiotic susceptibility pattern of Shiga toxin-producing *Escherichia coli* (STEC) from cattle and camels in Maiduguri, North-Eastern Nigeria. Trop Anim Health Prod. 2018;50(6):1327-41. doi: 10.1007/s11250-018-1565-z.

337. Stålsby Lundborg C, Tamhankar AJ. Understanding and changing human behaviour—antibiotic mainstreaming as an approach to facilitate modification of provider and consumer behaviour. Ups J Med Sci. 2014;119(2):125-33. doi: 10.3109/03009734.2014.905664.

338. Knobler SL LS, Najafi M, et al., editors. The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary. Washington (DC): National Academies Press (US); 2003. 5, Factors Contributing to the Emergence of Resistance. Institute of Medicine (US) Forum on Emerging Infections. 2003.

339. Okeke IN, Lamikanra A, Edelman R. Socioeconomic and Behavioral Factors Leading to Acquired Bacterial Resistance to Antibiotics in Developing Countries. Emerg Infect Dis. 1999;5(1):18-27. doi: 10.3201/eid0501.990103.

340. Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiology Reviews. 2018;42(1):fux053. doi: 10.1093/femsre/fux053.

341. Woodford N, Ellington MJ. The emergence of antibiotic resistance by mutation. Clin Microbiol Infect. 2007;13(1):5-18. doi: https://doi.org/10.1111/j.1469-0691.2006.01492.x.

342. Furuya EY, Lowy FD. Antimicrobial-resistant bacteria in the community setting. Nat Rev Microbiol. 2006;4:36. doi: 10.1038/nrmicro1325.

343. Gopal Rao G. Risk Factors for the Spread of Antibiotic-Resistant Bacteria. Drugs. 1998;55(3):323-30. doi: 10.2165/00003495-199855030-00001.

344. Centers for Disease Control and Prevention. Antibiotic Use in the United States, 2017: Progress and Opportunities. 2017.

345. Infectious Diseases Society of America. Combating Antimicrobial Resistance: Policy Recommendations to Save Lives. Clin Infect Dis. 2011;52(Suppl 5):S397-S428. doi: 10.1093/cid/cir153.

346. Antibiotic Resistance Action Center. U.S. Policies and Resources. 2018.

347. Munir M, Wong K, Xagoraraki I. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. Water Res. 2011;45(2):681-93. doi: 10.1016/j.watres.2010.08.033.

348. Walia S, Murleedharn C, Band J, Kanwar M, Kumar A. Quantitation of antibiotic resistance genes pollution in hospital waste water effluent and Urban Clinton River Water, Michigan, USA. Curr Med Res Prac. 2016;6(4):149-51. doi: https://doi.org/10.1016/j.cmrp.2016.07.005.

349. Schnabel EL, Jones AL. Distribution of Tetracycline Resistance Genes and Transposons among Phylloplane Bacteria in Michigan Apple Orchards. Appl Environ Microbiol. 1999;65(11):4898.

CHAPTER 2

Frequency and Epidemiologic Factors Associated with Clinical Antibiotic Resistant Shiga Toxin-producing *Escherichia coli* (STEC) Infections in Michigan, 2010-2014

This chapter is from a published manuscript:

Mukherjee S, Mosci RE, Anderson CM, Snyder BA, Collins J, Rudrik JT, et al. Antimicrobial Drug–Resistant Shiga Toxin–Producing *Escherichia coli* Infections, Michigan, USA. *Emerg Infect Dis*. 2017;23(9):1609-1611. https://dx.doi.org/10.3201/eid2309.170523

ABSTRACT

STEC is a serious health burden in the US, resulting in approximately 265,000 food infections every year. Although the Centers for Disease Control and Prevention (CDC) does not recommend the use of antimicrobial agents for the treatment of STEC infections, antibiotic resistance has emerged in both STEC O157 and non-O157 serotypes worldwide. Since STEC are widespread in the environment, the possibility of lateral transfer of resistance genes to other clinically relevant bacteria adds to the threat of antibiotic resistance. Here, we characterized 358 STEC isolates collected from patients in Michigan as part of the Enterics Research Investigational Network (ERIN) surveillance system between 2010 and 2014. Since antibiotic resistance in STEC has not been widely researched, we sought to determine the frequencies of antibiotic resistance to clinically relevant antibiotics and identify factors associated with resistant infections. Although antibiotic resistance was more common in non-O157 strains, high frequencies of antibiotic resistance were observed in both O157 (5.5%) and non-O157 (11.1%) STEC strains. Antibiotic resistance was also independently associated with hospitalizations (Odds Ratio (OR): 2.4; 95% Confidence Interval (CI): 1.00, 5.82) indicating that resistance could contribute to more severe disease outcomes. These findings highlight the need for continuous surveillance of antibiotic resistance and the identification of targeted intervention strategies to reduce the burden of antibiotic resistant STEC in Michigan.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC), a leading cause of foodborne illness in the USA, contributes to 265,000 cases, 3600 hospitalizations and 30 deaths annually (1). In the US, most infections are caused by O157 strains, however, increases in non-O157 STEC have been documented (2). Indeed, until the year 2000, non-O157 serotypes were not nationally notifiable (3) and the increase in incidence of non-O157 infections may be attributed to changes in laboratory surveillance and detection. Of the non-O157 isolates, six non-O157 serotypes (O26, O103, O111, O121, O45 and O145) referred to as the 'big six' are particularly problematic in the US, accounting for 71% of all non-O157 infections in the US (3). The incidence of non-O157 STEC cases increased from 0.12 to 0.95 per 100,000 between 2000 and 2010, while the incidence of O157 STEC cases decreased from decreased from 2.17 to 0.95 per 100,000 during the same time period (4). Furthermore, in 2014 alone, 690 non-O157 cases (1.43 per 100,000) and 445 O157 cases (0.92 per 100,000) were reported in the US; O157 serotypes were found to result in more hospitalizations and deaths when compared to non-O157 serotypes (5).

Animals such as cattle, pigs and sheep serve as reservoirs of STEC (6-8); cattle are considered to be an important reservoir with STEC frequently isolated from cattle and beef products (8-11). Since animals are reservoirs of STEC, animal contact is a major source of STEC infections in humans. Indeed, occupational and recreational contact with animals has been identified as a major risk factor of STEC infections in humans (12-14). Other sources of infection include consumption of contaminated food and water (11, 15-17).

Pathogenic STEC are characterized by the presence of *stx* genes which encode the Shiga toxin, a primary virulence factor. Shiga toxins are classified as Stx1 or Stx2 which are antigenically unrelated and can be further classified into subtypes and variants (18, 19). Importantly, the

presence of *stx2* has been observed to be significantly associated with increased risk of severe disease outcomes such as hemolytic uremic syndrome (HUS) (3, 20). In addition, pathogenic STEC may also contain the locus of enterocyte effacement (LEE) pathogenicity island carrying the *eae* gene, encoding the intimin protein, which is essential for the formation of the attaching and effacing (A/E) lesions on intestinal mucosa (21-24).

STEC infections are usually self-limiting resulting in watery diarrhea, nausea, abdominal pain and vomiting. However, STEC infections can progress to severe infections resulting in hemorrhagic colitis and hemolytic uremic syndrome (HUS) (25). While antibiotics are not recommended for the treatment of STEC infections due to the risk of development of HUS (26, 27), antibiotics have been found to be prescribed to patients with STEC infections (28). Indeed, antibiotic resistance among STEC has been reported (29-31), though the frequency is likely underestimated because susceptibility testing is not routine and not all laboratory-confirmed cases yield isolates for testing.

The FoodNet active surveillance system tracks the incidence of important food pathogens in the US while the National Antimicrobial Resistance Monitoring System (NARMS) monitors trends in antibiotic resistance. Since Michigan is not one of the ten states included in the FoodNet surveillance system, information on the incidence and factors associated with STEC infections in Michigan is lacking. Also, while NARMS conducts antimicrobial susceptibility testing for O157 isolates, it does not do so for the non-O157 serotypes. Given the importance of resistance in other *E. coli* pathotypes, we sought to determine the prevalence of resistant STEC O157 and non-O157 infections and assess the impact of resistance on disease.

MATERIALS AND METHODS

Study population and STEC isolates

A total of 358 laboratory confirmed STEC cases reported to the Michigan Department of Health and Human Services (MDHHS) between January 1, 2010 and December 31, 2014. A subset of these isolates was collected as part of the Enterics Research Investigational Network (ERIN) surveillance system, which was set up in collaboration with the MDHHS and four hospitals: Sparrow Health System, Detroit Medical Center, Spectrum Health Systems and University of Michigan Medical Center in Michigan. All protocols used in this study were approved by the Institutional Review Boards at Michigan State University (MSU; Lansing, MI, USA; IRB #10-736SM) and the MDHHS (842-PHALAB). The serotypes O157, O45, O103, O111 and O26 were studied because they are the most frequent cause of gastrointestinal disease in the USA. Isolates were grown at 37^{o}C in Luria-Bertani (BD Diagnostics) media under aerobic conditions for 18-20h and were stored in Luria-Bertani broth with 10% glycerol at -80°C until further testing. Molecular serotypes were confirmed using PCR and each strain was examined for the presence of *eae* and *stx* (32).

Antimicrobial susceptibility testing

All strains were examined for resistance to three clinically important antibiotics: ampicillin (10 µg), trimethoprim-sulfamethoxazole/SXT (25 µg) and ciprofloxacin (5 µg) using the Kirby-Bauer disc diffusion test on Mueller Hinton agar plates (33); the minimum inhibitory concentration (MIC) was determined using ETest® bioMérieux. After an incubation period of 18-20 hours at 37^{o}C, the zone of clearance around each Oxoid™ antibiotic disc was measured in millimeters. Strains were classified as resistant or susceptible as recommended by the Clinical Laboratory

Standards Institute (CLSI) (34) and the laboratory reference strain, *E. coli* ATCC 25922, was used as the quality control organism. If isolates were resistant to all three antibiotics, they were defined as being multidrug resistant. Only one strain was selected for antibiotic susceptibility testing per outbreak to avoid overestimating antibiotic resistance frequencies.

Epidemiological data

Epidemiological data and demographic data were collected from the Michigan Disease Surveillance System (MDSS) and managed using Microsoft Excel and Access. Season was classified as winter (December, January, and February), spring (March, April, and May), summer (June, July, and August) and fall (September, October, and November) based on when the case was reported; for those cases with a missing date, the date of isolation of the pathogen was used. Based on the classification scheme by the National Center for Health Statistics (NCHS) ten Michigan counties were classified as urban while the rest were classified as rural (35). Counties were also classified as high (prescribing rates 30% higher than the state average) or low antibiotic users based on the report by the Center for Healthcare Research and Transformation (CHRT) (36). The stratification by age group used in this study was based on reports that incidence of STEC cases were lowest among adults between 18-59 years of age (4) and age groups used by CDC to describe antibiotic use in the United States (37).

Data analysis

All statistical analyses were carried out using SAS version 9.4 (SAS Institute, Cary, NC, USA) and Epi Info™ 7. Chi-square test and Fisher's exact test were used to determine significant associations between dependent and independent variables; a p value ≤ 0.05 was considered significant. Variables found to have strong associations with antibiotic resistance in the univariate

analysis (p value $\leq$ 0.20) were included in the multivariate analysis. Multivariate analysis, which included potentially confounding factors such as age and sex, was carried out using forward logistic regression to build a model containing significant variables (p value $\leq$ 0.05) independently associated with antibiotic resistance. The Mantel-Haenszel $\chi 2$ test was used for analyzing trends, with a p value $\leq$ 0.05 considered to be significant.

RESULTS

Description of STEC cases in Michigan

Between January 2010 and December 2014, a subset of 358 STEC isolates were recovered from patients in Michigan. The characteristics of these cases are described in Table 2.1. Among the 358 isolates examined, 41.4% (n=146) were serotyped as O157 while 58.6% (n=207) were non-O157 isolates; the serotype was unknown for 5 isolates. Among the 207 non-O157 isolates, 24.1% (n=50) were O45, 36.2% (n=75) were O103, 14.0% (n=29) were O111 and 25.6% (n=53) were O26. On examining the *stx* gene profiles of all isolates, most STEC isolates carried the *stx1* gene (n=205, 57.3%), while 20.9% (n=75) carried *stx2* gene and 21.5% (n=77) carried both *stx1* and *stx2* genes. The *stx* gene profile was missing for one isolate. Of the 358 cases, 48.3% (n=173) were male while 51.7% (n=185) were female. The highest frequency of STEC cases was observed in adults between the ages of 19 and 52 years (n=141, 39.4%). Abdominal pain (n=279, 83.0%) and diarrhea (n=271, 80.6%) were the most commonly reported symptoms. While 30.9% (n=106) cases were hospitalized, only six (1.8%) cases developed HUS.

Antimicrobial susceptibility profiles and trends in antimicrobial resistance

STEC isolates from 2010-2014 were also found to be resistant to antibiotics; 32 (8.9%) were resistant to at least one antibiotic, 27 (7.5%) were resistant to ampicillin, 15 (4.2%) were

resistant to trimethoprim-sulfamethoxazole and only one (0.3%) were resistant to ciprofloxacin. When stratified by serotypes, one isolate with an unknown serotype was found to be resistant to both ampicillin and trimethoprim-sulfamethoxazole. Of the 353 isolates for which serotypes were known, 31 (8.8%) strains (23 non-O157, 8 O157) were resistant to antimicrobial drugs (Table 2.2); resistance to ampicillin (7.4%) was most common, followed by trimethoprim-sulfamethoxazole (4.0%) and ciprofloxacin (0.3%). One strain was resistant to all drugs (Table 2.3), and all resistant strains had high minimum inhibitory concentrations (MICs) (ampicillin, >64 μg/mL; ciprofloxacin, >32 μg/mL; SXT, in 1:19 ratio, >32/608 μg/mL). Notably, resistance was twice as common for non-O157 (11.1%) than for O157 (5.5%) strains (Table 2.4). The O111 serotypes (n = 7) had significantly higher resistance frequencies (24.1%) than other non-O157 serogroups (p = 0.03).

Although no significant difference was observed by *stx* profile (Table 2.4), strains possessing *stx*1 only were more commonly resistant than strains with *stx*2 alone (Fisher exact test *p* value = 0.27). All 23 (100%) resistant non-O157 STEC and 1 (12.5%) resistant O157 strain had *stx*1 only. Strains positive for *eae* were less likely to be resistant (n = 27; 8.4%) than *eae*-negative strains (n = 4; 23.5%) (Table 2.4); this nonsignificant difference (Fisher exact test *p* value = 0.07) could be due to small sample sizes. Variation was also observed by serotype as all eight (100%) of the resistant O157 strains had *eae* similar to 18 of the 22 (81.8%) resistant non-O157 strains.

Fluctuating frequencies in resistance were observed between 2010-2014 with a marked increase among STEC O157 strains recovered in 2012, although not significant (Figure 2.1). Importantly, resistance to ampicillin and trimethoprim-sulfamethoxazole was higher for the STEC O157 strains recovered in Michigan relative to the national rates reported by the National Antimicrobial Resistance Monitoring System (NARMS) (38), although this difference was not

significant (Figure 2.2). Interestingly, an increase in frequencies of resistance to ampicillin and trimethoprim-sulfamethoxazole in STEC O157 isolates from 2011 to 2012 was observed, however, this difference was not significant (Figure 2.3).

Epidemiological associations with antibiotic resistant STEC infections

To determine whether antibiotic resistant infections are associated with severe disease outcomes, a multivariate analysis was conducted using logistic regression with hospitalization as the dependent variable. Variables with significant ($p \leq 0.05$) and strong associations ($p \leq 0.20$) from the univariate analysis were included as independent variables. Forward selection identified resistance (OR:2.4; 95% CI:1.00, 5.82) to be associated with hospitalization (Table 2.5), suggesting that resistant infections may cause more severe clinical outcomes. Patients ≥18 years, women, and those presenting with bloody diarrhea were also more likely to be hospitalized, while non-O157 infection was protective (OR: 0.4; 95% CI: 0.21, 0.61).

We also observed higher frequencies of resistance in counties with high antibiotic prescription rates compared to those with lower rates, although this difference was not significant (Table 2.4). Higher frequencies of antibiotic resistance were also observed in winter and spring compared to summer and fall, although this difference was not significant (Table 2.4).

DISCUSSION

Overall, we detected a high frequency of resistance among non-O157 STEC (11.1%) in this population, which is similar to findings from other studies although fewer antibiotics were tested herein (30, 31, 39, 40). We also observed higher frequencies of resistance in clinical non-O157 serotypes compared to O157 serotypes, which has also been reported previously (30). Mora et al. reported higher frequencies of resistance in non-O157 STEC (47%) than in O157 STEC (23%)

isolates from humans (30). Interestingly, higher frequencies of resistance were noted in bovine O157 STEC (53%) than the non-O157 serovars (38%) in the same study (30). In contrast, Sasaki et al. reported higher frequencies of antibiotic resistance in STEC O26 serotypes (54.5%) than in STEC O157 serotypes (13.3%) in isolates from beef cattle in Japan, although, the small sample size of STEC O26 isolates may be influencing resistance frequencies (41). These studies suggest that antibiotic resistance frequencies among STEC O157 and non-O157 isolates may differ by the source of the isolates. Interestingly, ciprofloxacin resistance was low despite its routine use for treatment of enteric infections and may be because multiple mutations are required in *E. coli* to achieve clinical resistance (42). Relative to other *E. coli* pathotypes such as extraintestinal *E. coli*, however, resistance frequencies in STEC were low, which may be attributable to differences in the source of these distinct infections (29). Furthermore, higher O157 resistance frequencies of resistance in Michigan versus the nation also indicate that selection pressures vary by location and source. Although no difference was observed in resistance frequencies for counties with high versus low antibiotic prescription rates (36), we have not investigated selection pressures from antibiotic use in the farm environment that may impact resistance emergence in Michigan. Approximately $12x10^6$ kg of antibiotics including $5x10^6$kg of tetracycline, $6.9x10^5$kg of penicillin and $5x10^5$kg of sulfa drugs, are administered to food animals annually in the USA; ~61% of these antibiotics are medically relevant and useful (43). The higher resistance frequencies observed in winter and spring (12.2%) versus summer and fall (7.4%) could also be attributed to variation in prescription rates by season (44). In this study, antibiotic resistant STEC infections were also found to be associated with hospitalizations, a marker of more severe disease outcomes. While our study is the first to observe an association between antibiotic resistant STEC and hospitalization, prior studies have documented a link between resistance and severe disease. For example, Varma et al.

observed antibiotic resistant non-typhoidal *Salmonella* infections to be associated with bloodstream infections and hospitalizations (45), while a significant increase in mortality was associated with methicillin resistant *Staphylococcus aureus* (MRSA) than when patients were infected with methicillin susceptible *S. aureus* (MSSA) (46).

Since Michigan is not included in FoodNet and resistance in STEC has not been widely researched, data about the prevalence and impact of resistance is lacking. This study detected a high frequency of STEC resistance to antibiotics commonly used in human and veterinary medicine, particularly for non-O157 serotypes, which have increased in frequency (2). Monitoring resistance in STEC is essential because of the risk of transmitting resistant strains from food animals to humans and the high likelihood of horizontal transfer of resistance genes from STEC to other pathogens. Moreover, the high level of resistance to antibiotics important in human and veterinary medicine among non-O157 STEC strains is concerning because of its importance as a leading cause of foodborne infections. Because of the negative health outcomes associated with resistance, routine monitoring can also potentially uncover new treatment approaches and guide the development of strategies for controlling the emergence and spread of resistance in STEC and other *E. coli* pathotypes.

APPENDIX

Table 2.1. Descriptive study of Shiga toxin-producing *E. coli* (STEC) cases in Michigan (2010-2014)

Characteristic	No. of cases‡	Percentage (%) of cases
Demographic data		
Sex		
Male	173	48.3%
Female	185	51.7%
Age group (years)		
≤ 2	31	8.7%
3 – 10	57	15.9%
11 – 18	66	18.4%
19 – 52	141	39.4%
≥53	63	17.6%
Race		
Caucasian	291	90.9%
African American	16	5.0%
Other or Mixed Race	13	4.1%
Ethnicity		
Hispanic or Latino	13	4.8%
Not Hispanic or Latino	259	95.2%
Residence (counties in Michigan)		
Wayne	27	7.5%
Kent	31	8.7%
Oakland	33	9.2%
Ingham	6	1.7%
Ottawa	13	3.6%
Macomb	24	6.7%
Kalamazoo	5	1.4%
Washtenaw	17	4.7%
Others	202	56.4%
Pathogen data		
Serotype		
O157	146	41.4%
Non-O157	207	58.6%
***stx* gene**		
*stx*1	205	57.3%
*stx*2	75	20.9%
*stx*1*stx*2	77	21.5%
Clinical Outcomes		
Case hospitalization	106	30.9%
Abdominal pain	279	83.0%
Body ache	55	16.4%
HUS	6	1.8%

Table 2.1 (cont'd)

Bloody diarrhea	232	69.0%
Diarrhea	271	80.6%

The percentages are based on the number of cases for which information was available. Counts for sex, age group and race are mutually exclusive for each category. Counts for animal contact and food consumption are repeated across categories.

‡ Total number of cases varies between variables due to the difference in missing information.

Table 2.2. Antimicrobial drug resistance in 353 clinical Shiga toxin-producing *E. coli* (STEC) isolates, by serotype in Michigan, 2010–2014. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

		Any resistance		AMP resistance		CIP resistance		SXT resistance	
Serotype	No. isolates‡	No.	(%)	No.	(%)	No.	(%)	No.	(%)
O157	146	8	(5.5)	7	(4.8)	0	(0)	5	(3.4)
Non-O157	207	23	(11.1)	19	(9.2)	1	(0.5)	9	(4.3)
O26	53	4	(7.6)	4	(7.6)	0	(0)	1	(1.9)
O45	50	6	(12.0)	5	(10.0)	0	(0)	2	(4.0)
O103	75	6	(8.0)	5	(6.7)	1	(1.3)	4	(5.3)
O111	29	7	(24.1)	5	(17.2)	0	(0)	2	(6.9)

Isolate numbers for individual antibiotics do not always add up to the total number of isolates with any resistance because some isolates were resistant to more than one antibiotic.

‡ Five isolates had unknown serotypes and were excluded from this analysis.

Table 2.3. Multidrug resistance in 358 clinical Shiga toxin-producing *E. coli* (STEC) in Michigan, 2010-2014

Resistance Pattern	No. (%) of Resistant Strains (n=358)
No resistance detected	326 (91.1%)
Resistance to at least one antibiotic class	32 (8.9%)
Resistance to only 1 antimicrobial class	22 (6.1%)
Resistance to only 2 antimicrobial classes	9 (2.5%)
Resistance to all 3 antimicrobial classes	1 (0.3%)

Table 2.4. Univariate analysis highlighting factors associated with antibiotic resistance in 358 clinical Shiga toxin-producing *E. coli* (STEC) in Michigan, 2010-2014

Characteristic	Total strains*	No (%) resistance	OR (95% CI†)	*p* value‡
Pathogen factors				
Serotype				
O157	146	8 (5.5%)	1.0	-
Non-O157	207	23 (11.1%)	2.2 (0.94-4.97)	0.066
stx profile				
*stx*1	205	25 (12.2%)	1.9 (0.72-5.28)	0.18
*stx*2	75	5 (6.7%)	1.0	-
stx1,*stx2*	77	2 (2.6%)	0.3 (0.07-1.99)	0.27
eae presence				
Yes	323	27 (8.4%)	0.3 (0.10-1.04)	0.05
No	18	4 (22.2%)	1.0	-
Outbreak associated				
Yes	14	1 (7.1%)	0.8 (0.10-6.14)	0.81
No	344	31 (9.0%)	1.0	-
Demographics and other factors				
Residence				
Urban	153	13 (8.5%)	0.9 (0.43-1.90)	0.80
Rural	205	19 (9.3%)	1.0	-
Age in years				
0-18	154	12 (7.8%)	1.0	-
19-64	172	17 (9.9%)	1.3 (0.60-2.81)	0.51
≥ 65	32	3 (9.4%)	1.2 (0.32-4.61)	0.76
Sex				
Male	173	14 (8.1%)	1.0	-
Female	185	18 (9.7%)	1.2 (0.59-2.54)	0.59
Antibiotic Prescription rates by county	109	13 (11.9%)	1.6 (0.78-3.45)	0.19
High	249	19 (7.6%)	1.0	-
Low				
Season				
Winter and Spring	115	14 (12.2%)	1.7 (0.83-3.62)	0.14
Summer and Fall	243	18 (7.4%)	1.0	-
Clinical factors				
Abdominal pain				
Yes	279	27 (9.7%)	1.4 (0.48-4.23)	0.53
No	57	4 (7.0%)	1.0	-

Table 2.4 (cont'd)

Body ache				
Yes	55	7 (12.7%)	1.6 (0.64-3.83)	0.33
No	281	24 (8.5%)	1.0	-
Bloody diarrhea				
Yes	232	21 (9.1%)	0.9 (0.42-2.06)	0.87
No	104	10 (9.6%)	1.0	-
Hemolytic uremic syndrome (HUS)	6	0 (0%)	--	1.0
Yes	331	31 (9.4%)		
No				
Hospitalization				
Yes	106	13 (12.3%)	1.7 (0.80-3.61)	0.16
No	237	18 (7.6%)	1.0	-

*Depending on the variable examined, the number of isolates does not add up to the total (n=358) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells

Table 2.5. **Univariate analysis to identify factors associated with hospitalization**

Characteristic	**Total strains***	**No (%) hospitalized**	**OR (95% CI)†**	***p* value‡**
Serotype				
O157	138	63 (45.7%)	1.0	-
Non-O157	200	42 (21.0%)	0.3 (0.20-0.51)	<0.0001
stx profile				
*stx*1	198	43 (21.7%)	0.3 (0.18-0.58)	<0.0001
*stx*2	72	33 (45.8%)	1.0	-
stx1,*stx2*	72	30 (41.7%)	1.7 (0.86-3.20)	0.13
eae presence				
Yes	310	92 (29.7%)	0.5 (0.20-1.50)	0.23
No	16	7 (43.8%)	1.0	-
Outbreak associated				
Yes	14	7 (50.0%)	2.5 (0.79-6.80)	0.11
No	329	99 (30.1%)	1.0	-
Antibiotic resistant isolate				
Yes	31	13 (41.9%)	1.7 (0.80-3.61)	0.16
No	312	93 (29.8%)	1.0	-
Sex				
Male	166	39 (23.5%)	1.0	-
Female	177	67 (37.9%)	2.0 (1.24-3.17)	0.004
Age in years				
0-18	145	35 (24.1%)	1.0	-
18-64	167	56 (33.5%)	1.6 (0.96-2.61)	0.07
≥ 65	31	15 (48.4%)	2.9 (1.32- 6.56)	0.007
Abdominal pain				
Yes	277	95 (34.3%)	2.2 (1.08-4.41)	0.03
No	57	11 (19.3%)	1.0	-
Body ache				
Yes	55	20 (36.4%)	1.3 (0.70-2.35)	0.42
No	279	86 (30.8%)	1.0	-
Bloody diarrhea				
Yes	230	91 (39.6%)	3.9 (2.12-7.13)	<0.0001
No	104	15 (14.4%)	1.0	-
Hemolytic uremic syndrome (HUS)				
Yes	5	4 (80.0%)	9.0 (0.99-81.45)	0.02
No	328	101 (30.8%)	1.0	-

Table 2.5 (cont'd)

* Depending on the variable examined, the number of isolates does not add up to the total (n=358) because of missing data. All 6 HUS cases had O157 strains with *eae*, though 3 had *stx1*, *stx2* and the other 3 had *stx2* infections

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells.

Table 2.6. Multivariate analysis to identify factors associated with hospitalization

	Multivariate logistic regression associations £		
Characteristic	**OR**	**95% CI €**	***p* value**
Sex: Female	1.9	1.15-3.32	0.02
Age in years: ≥ 18	1.9	1.15-3.28	0.014
Serogroup: non-O157	0.4	0.21-0.61	0.0002
Antibiotic Resistant Isolate: Yes	2.4	1.00-5.82	0.05
Bloody Diarrhea: Yes	3.9	1.99-7.65	<0.0001

£ Logistic regression was performed using forward selection while controlling for variables that yielded significant (P≤0.05) and strong (P≤0.20) associations with hospitalization in the univariate analysis. The model was adjusted for age, sex, serogroup, *stx* profile, outbreak status, resistance, HUS, and bloody diarrhea. Only those variables yielding significant associations are presented; Hosmer and Lemeshow Goodness-of-Fit test (P= 0.73). All variables were tested for collinearity.

€ Wald 95% confidence intervals (CI)

Figure 2.1. Trends in resistance to at least one antibiotic (ampicillin, ciprofloxacin and trimethoprim-sulfamethoxazole) among O157 and non-O157 Shiga toxin-producing *E. coli* (STEC) isolates recovered from patients in Michigan, 2010–2014

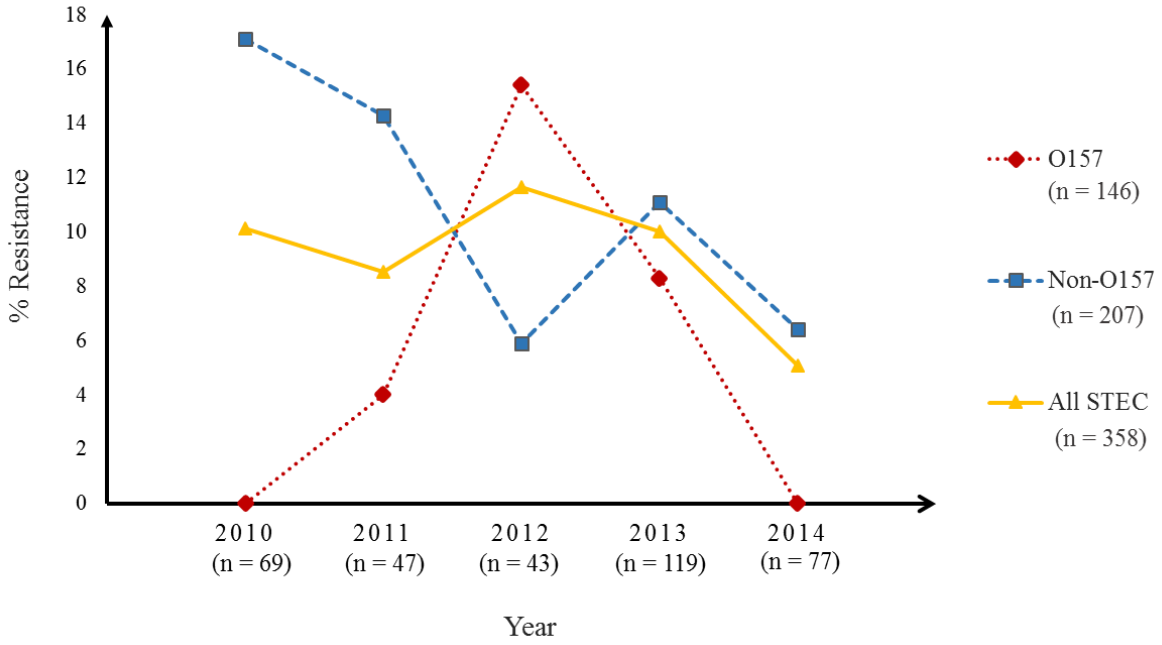


Figure 2.2. Comparison between Shiga toxin-producing *E. coli* (STEC) isolates from Michigan and National Antimicrobial Resistance Monitoring System (NARMS), 2010-2014. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

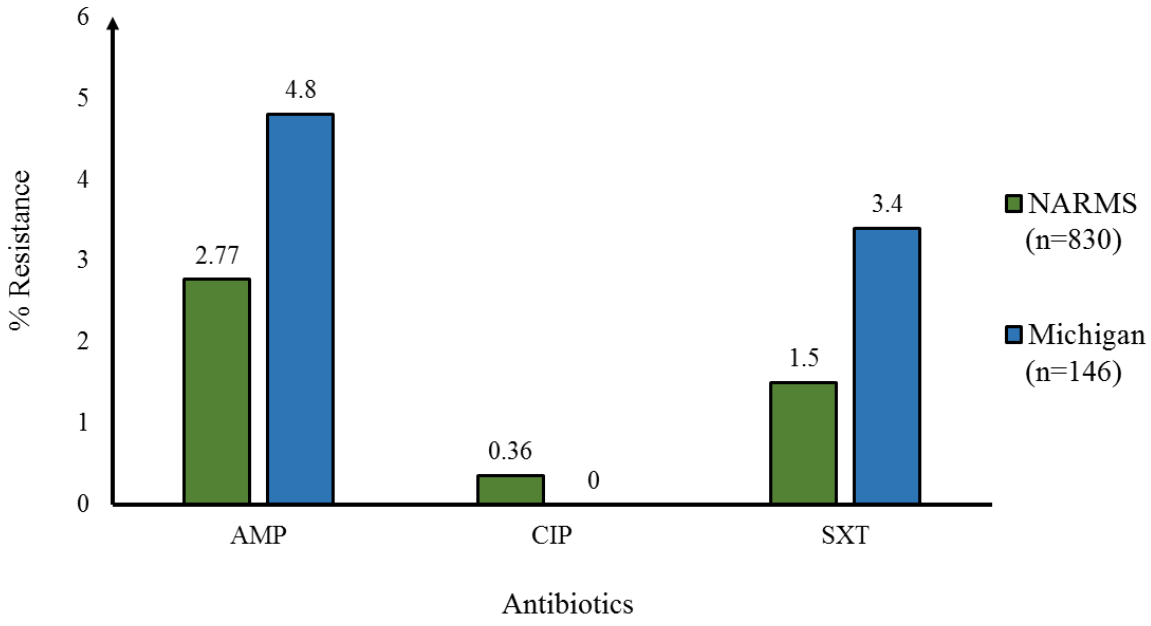
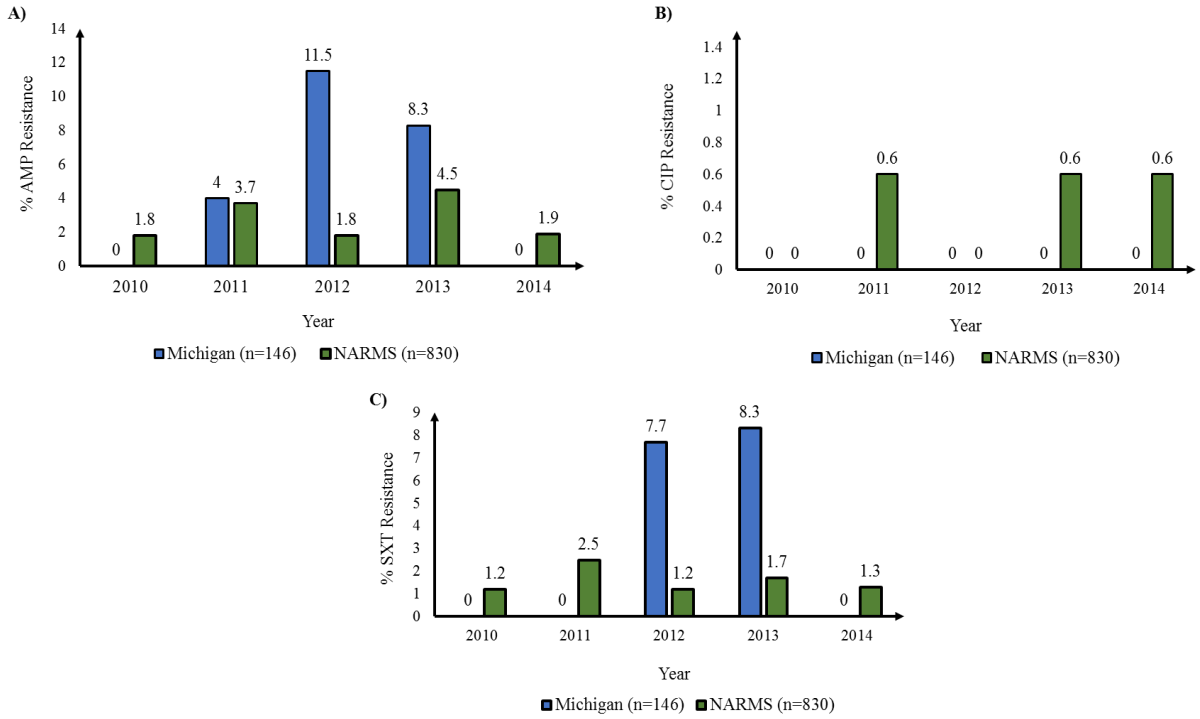


Figure 2.3. Trends in antibiotic resistance in Shiga toxin-producing *E. coli* (STEC) O157 isolates from Michigan and those tested by National Antimicrobial Resistance Monitoring System (NARMS), 2010-2014. A) % frequency resistance to ampicillin by year, B) % frequency of resistance to ciprofloxacin by year, C) % frequency of resistance to trimethoprim-sulfamethoxazole by year. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole



REFERENCES

REFERENCES

1. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1.

2. Tseng M, Sha Q, Rudrik JT, Collins J, Henderson T, Funk JA, Manning SD. Increasing incidence of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in Michigan and association with clinical illness. Epidemiol Infect. 2016;144(7):1394-405. doi: 10.1017/S0950268815002836.

3. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. J Infect Dis. 2005;192(8):1422-9. doi: 10.1086/466536.

4. Gould LH, Mody RK, Ong KL, Clogher P, Cronquist AB, Garman KN, Lathrop S, Medus C, Spina NL, Webb TH, White PL, Wymore K, Gierke RE, Mahon BE, Griffin PM for Emerging Infections Program Foodnet Working Group. Increased recognition of non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States during 2000-2010: epidemiologic features and comparison with *E. coli* O157 infections. Foodborne Pathog Dis. 2013;10(5):453-60. doi: 10.1089/fpd.2012.1401.

5. Crim SM, Griffin PM, Tauxe R, Marder EP, Gilliss D, Cronquist AB, Cartter M, Tobin-D'Angelo M, Blythe D, Smith K, Lathrop S, Zansky S, Cieslak PR, Dunn J, Holt KG, Wolpert B, Henao OL. Preliminary incidence and trends of infection with pathogens transmitted commonly through food - Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2014. MMWR Morb Mortal Wkly Rep. 2015;64(18):495-9.

6. Zschock M, Hamann HP, Kloppert B, Wolter W. Shiga-toxin-producing *Escherichia coli* in faeces of healthy dairy cows, sheep and goats: prevalence and virulence properties. Lett Appl Microbiol. 2000;31(3):203-8.

7. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F. Prevalence and some properties of Verotoxin (Shiga-Like Toxin)- producing *Escherichia coli* in 7 different species of healthy domestic animals. J Clin Microbiol. 1993;31(9):2483-8.

8. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvirta T, Pelkonen S, Yamamoto K. Prevalence and characteristics of shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl Environ Microbiol. 2001;67(1):484-9. doi: 10.1128/AEM.67.1.484-489.2001.

9. Hussein HS, Bollinger LM. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. J Food Prot. 2005;68(10):2224-41.

10. Cobbold RN, Rice DH, Szymanski M, Call DR, Hancock DD. Comparison of shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in Washington State. Appl Environ Microbiol. 2004;70(7):4375-8.

11. Brusa V, Aliverti V, Aliverti F, Ortega EE, de la Torre JH, Linares LH, Sanz ME, Etcheverria AI, Padola NL, Galli L, Peral Garcia P, Copes J, Leotta GA. Shiga toxin-producing *Escherichia coli* in beef retail markets from Argentina. Front Cell Infect Microbiol. 2012;2:171. doi: 10.3389/fcimb.2012.00171.

12. Schlager S, Lepuschitz S, Ruppitsch W, Ableitner O, Pietzka A, Neubauer S, Stöger A, Lassnig H, Mikula C, Springer B, Allerberger F. Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. Int J Med Microbiol. 2018; 308(7):927-932. doi: https://doi.org/10.1016/j.ijmm.2018.06.008.

13. Rivas M, Sosa-Estani S, Rangel J, Caletti MG, Valles P, Roldan CD, Balbi L, Marsano de Mollar MC, Amoedo D, Miliwebsky E, Chinen I, Hoekstra RM, Mead P, Griffin PM. Risk factors for sporadic Shiga toxin-producing *Escherichia coli* infections in children, Argentina. Emerg Infect Dis. 2008;14(5):763-71. doi: 10.3201/eid1405.071050.

14. McPherson M, Lalor K, Combs B, Raupach J, Stafford R, Kirk MD. Serogroup-specific risk factors for Shiga toxin-producing *Escherichia coli* infection in Australia. Clin Infect Dis. 2009;49(2):249-56.

15. Mohammadi P, Abiri R, Rezaei M, Salmanzadeh-Ahrabi S. Isolation of Shiga toxin-producing *Escherichia coli* from raw milk in Kermanshah, Iran. Iran J Microbiol. 2013;5(3):233-8.

16. Slayton RB, Turabelidze G, Bennett SD, Schwensohn CA, Yaffee AQ, Khan F, Butler C, Trees E, Ayers TL, Davis ML, Laufer AS, Gladbach S, Williams I, Gieraltowski LB. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. PLoS One. 2013;8(2):e55300. doi: 10.1371/journal.pone.0055300.

17. Lascowski KM, Guth BE, Martins FH, Rocha SP, Irino K, Pelayo JS. Shiga toxin-producing *Escherichia coli* in drinking water supplies of north Parana State, Brazil. J Appl Microbiol. 2013;114(4):1230-9.

18. Scheutz F, Teel LD, Beutin L, Pierard D, Buvens G, Karch H, Mellmann A, Caprioli A, Tozzoli R, Morabito S, Strockbine NA, Melton-Celsa AR, Sanchez M, Persson S, O'Brien AD. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J Clin Microbiol. 2012;50(9):2951-63.

19. Scheutz F. Taxonomy Meets Public Health: The Case of Shiga Toxin-Producing *Escherichia coli*. Microbiol Spectr. 2014;2(3).

20. Jenkins C, Willshaw GA, Evans J, Cheasty T, Chart H, Shaw DJ, Frankel G, Smith HR. Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom. J Med Microbiol. 2003;52(Pt 11):941-7.

21. Etcheverria AI, Padola NL. Shiga toxin-producing *Escherichia coli*: factors involved in virulence and cattle colonization. Virulence. 2013;4(5):366-72.

22. Yu J, Kaper JB. Cloning and characterization of the *eae* gene of enterohaemorrhagic *Escherichia coli* O157:H7. Mol Microbiol. 1992;6(3):411-7.

23. Dean-Nystrom EA, Bosworth BT, Moon HW, O'Brien AD. *Escherichia coli* O157:H7 requires intimin for enteropathogenicity in calves. Infect Immun. 1998;66(9):4560-3.

24. Frankel G, Phillips AD, Trabulsi LR, Knutton S, Dougan G, Matthews S. Intimin and the host cell--is it bound to end in Tir(s)? Trends Microbiol. 2001;9(5):214-8.

25. Canpolat N. Hemolytic uremic syndrome. Turk Pediatri Ars. 2015;50(2):73-82.

26. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. N Engl J Med. 2000;342(26):1930-6.

27. Zhang X, McDaniel AD, Wolf LE, Keusch GT, Waldor MK, Acheson DW. Quinolone antibiotics induce Shiga toxin-encoding bacteriophages, toxin production, and death in mice. J Infect Dis. 2000;181(2):664-70.

28. Nelson JM, Griffin PM, Jones TF, Smith KE, Scallan E. Antimicrobial and antimotility agent use in persons with shiga toxin-producing *Escherichia coli* O157 infection in foodnet sites. Clin Infect Dis. 2011;52(9):1130-2.

29. Schroeder CM, Meng J, Zhao S, DebRoy C, Torcolini J, Zhao C, McDermott PF, Wagner DD, Walker RD, White DG. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerg Infect Dis. 2002;8(12):1409-14. doi: 10.3201/eid0812.020070.

30. Mora A, Blanco JE, Blanco M, Alonso MP, Dhabi G, Echeita A, Gonzalez EA, Bernardez MI, Blanco J. Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. Res Microbiol. 2005;156(7):793-806. doi: 10.1016/j.resmic.2005.03.006.

31. Amezquita-Lopez BA, Quinones B, Soto-Beltran M, Lee BG, Yambao JC, Lugo-Melchor OY, Chaidez C. Antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* O157 and Non-O157 recovered from domestic farm animals in rural communities in Northwestern Mexico. Antimicrob Resist Infect Control. 2016;5:1. doi: 10.1186/s13756-015-0100-5.

32. Singh P, Sha Q, Lacher DW, Del Valle J, Mosci RE, Moore JA, Scribner KT, Manning SD. Characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. Front Cell Infect Microbiol. 2015;5:29.

33. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology. 2009.

34. Clinical & Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

35. Ingram DD, Franco SJ. 2013 NCHS Urban-Rural Classification Scheme for Counties. Vital Health Stat 2. 2014(166):1-73.

36. Kofke-Egger H, Udow-Phillips M. Antibiotic Prescribing and Use. Center for Healthcare Research and Transformation,. 2011.

37. Centers for Disease Control and Prevention. Antibiotic Use in the United States, 2017: Progress and Opportunities. 2017.

38. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. 2018.

39. Khan A, Das SC, Ramamurthy T, Sikdar A, Khanam J, Yamasaki S, Takeda Y, Nair GB. Antibiotic resistance, virulence gene, and molecular profiles of Shiga toxin-producing *Escherichia coli* isolates from diverse sources in Calcutta, India. J Clin Microbiol. 2002;40(6):2009-15.

40. Meng J, Zhao S, Doyle MP, Joseph SW. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J Food Prot. 1998;61(11):1511-4.

41. Sasaki Y, Usui M, Murakami M, Haruna M, Kojima A, Asai T, Yamada Y. Antimicrobial resistance in Shiga toxin-producing *Escherichia coli* O157 and O26 isolates from beef cattle. Jpn J Infect Dis. 2012;65(2):117-21.

42. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005;41 Suppl 2:S120-6.

43. US Food and Drug Administration. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. 2011.

44. Suda KJ, Hicks LA, Roberts RM, Hunkler RJ, Taylor TH. Trends and seasonal variation in outpatient antibiotic prescription rates in the United States, 2006 to 2010. Antimicrob Agents Chemother. 2014;58(5):2763-6.

45. Varma JK, Molbak K, Barrett TJ, Beebe JL, Jones TF, Rabatsky-Ehr T, Smith KE, Vugia DJ, Chang HG, Angulo FJ. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J Infect Dis. 2005;191(4):554-61.

46. Cosgrove SE, Sakoulas G, Perencevich EN, Schwaber MJ, Karchmer AW, Carmeli Y. Comparison of Mortality Associated with Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Bacteremia: A Meta-analysis. Clin Infect Dis. 2003;36(1):53-9.

CHAPTER 3

Increasing Rates of Antibiotic Resistance in Shiga Toxin-Producing *Escherichia coli* (STEC) Infections in Michigan (2001-2014) and Factors Associated with Antibiotic Resistant Infections

ABSTRACT

STEC is an important foodborne pathogen resulting in approximately 1 million infections and 128 deaths every year, globally. In the US alone, STEC is reported to cause 265,000 illnesses every year, resulting in about 3,600 hospitalizations and 30 deaths. Although antibiotic resistance in STEC has not been widely researched, few studies have documented the emergence of antibiotic resistance in STEC. Periodic surveillance of antibiotic resistance is crucial for the development of informed policies to control the spread of antibiotic resistance. Thus, this study was undertaken to examine antibiotic resistance trends in clinical STEC isolates collected in Michigan for a period of 14 years, from 2001 to 2014. A total of 980 STEC isolates were collected as part of an active and a sentinel surveillance system in collaboration with the Michigan Department of Health and Human Service (MDHHS) and their antibiotic susceptibility profiles to clinically relevant antibiotics were determined. In addition, factors associated with antibiotic resistant infections were identified. Notably, increasing frequencies of antibiotic resistance in STEC isolates were observed during this 14-year period, with significantly higher frequencies observed in isolates from 2010-2014 (8.9%) than isolates from 2001-2009 (4.8%) (Chi Square *p* value=0.011). Multivariate logistic regression identified non-O157 serotypes as more likely to be resistant than O157 serotypes [Odds Ratio (OR): 2.3; 95% Confidence Interval (CI):1.36-3.96]. Among the O157 strains, those belonging to clade 8, a clinically important lineage, had higher resistance frequencies to more than one antibiotic compared to O157 isolates belonging to other clades (Fisher's exact *p* value =0.055). These findings demonstrate that antibiotic resistance in STEC isolates in Michigan is increasing over time, thus, highlighting the need for continued surveillance. Additionally, specific serotypes and phylogenetic lineages may play an important role in influencing resistance frequencies in different geographical areas.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) is an important foodborne pathogen causing an estimated 2,801,000 cases every year and results in 3890 cases of Hemolytic Uremic Syndrome (HUS) and 230 deaths, worldwide (1). In the US alone, STEC causes 265,000 illnesses every year, resulting in about 3,600 hospitalizations and 30 deaths (2). These data are obtained via FoodNet, an active surveillance system that monitors the incidence of STEC and other important foodborne pathogens in the US. According to FoodNet, the incidence rate of STEC per 100,000 population increased from 2.62 in 1996 to 3.02 in 2017 (Figure 3.1) (3). The annual incidence of confirmed STEC infections was highest in children <5 years of age (8.08 per 100,000 population), while the incidence was 3.95 and 2.87 per 1000,000 population for children between the ages of 5 and 9 years and 10 and 19 years, respectively (3). In the US, the annual incidence of STEC infections was higher in females (2.19 per 100,000 population) than males (1.9 per 100,000 population) (3); this trend has also been noted in other studies. Launders *et al*., for instance, reported a higher incidence of STEC O157 infections in females (1.77 per 100,000) than in males (1.4 per 100,000) in England (4). Additionally, the relative risk of STEC O157 infections females was 1.19 times the risk in males in England and Wales between 1983 and 2012 (5). While the reason for such differences by sex is unknown, consumption of higher quantities of fruits and vegetables by women (6) has been offered as a possible explanation since STEC infections have been linked with fruits and vegetables (7, 8). Similarly, it is well established that young children are highly susceptible to STEC infection (9) as well as long-term sequelae including HUS (10).

STEC strains have been classified into more than 472 serotypes (11) and variation in serotype prevalence has been documented by geographical region. Estimates from Europe suggest that non-O157 serotypes predominate causing up to 80% of diarrhea-associated STEC infections

(12-15). In North America, the O157 STEC serotypes have been more frequently reported than the non-O157 serotypes (16, 17); however, recent studies have shown an increased incidence of non-O157 serotypes in the US which is likely due to improved diagnostic procedures (17-19). The non-O157 serotypes of STEC were not nationally notifiable before the year 2000; however, at the request of the Council of State and Territorial Epidemiologists, public health laboratories in the US began reporting non-O157 STEC cases to the National Notifiable Diseases Surveillance System as well (20). In 2015, the incidence rate per 100,000 population was 1.65 for non-O157 STEC and 0.95 for O157 serotypes in the US (21). Between 1983-2002, the most commonly reported non-O157 serotypes causing infections in the US were O26 (22%), O111 (16%), O101 (12%), O121 (8%), O45 (7%) and O145 (5%) (20). Because Michigan is not one of the ten states included in the FoodNet Surveillance Network, the incidence and epidemiological associations are not available through the FoodNet Executive Report. However, in collaboration with the Michigan department of Health and Human Services (MDHHS), Tseng et al. reported the incidence of both STEC O157 and non-O157 infections in Michigan from 2001 to 2012, with the highest frequencies of cases belonging to O157:H7 (64.2%) and STEC O45:H2 (6.9%) serotypes (18). Importantly, a significant increase in the number of non-O157 outbreaks was observed (18), thus highlighting the importance and emergence of STEC non-O157 serotypes as human pathogens.

Seasonal variation in STEC cases has also been reported. For instance, Brooks et al. reported an increase in the number of STEC non-O157 isolates, collected between 1983-2002 in the US, between the months of June and September with a peak in incidence occurring in August (20). Additionally, in Michigan, most STEC infections reported between 2001 and 2012 occurred during the summer (37.3%) and autumn (29.5%) months (18). This consistent peak in infections during late summer and fall has also been observed in other enteric pathogens (22, 23) and may be

due to inadequate cooking and storage temperatures, at picnics and barbeques, which are more likely to take place during warmer months (24). Visiting state fairs and petting zoos which more common in summer and fall seasons (25) and have been linked to STEC infection previously (26), may also contribute to the seasonal variation observed. In addition, higher temperatures have been identified to be associated with increased STEC shedding in cattle (27). This high shedding of STEC by cattle during summer and fall may contaminate food and water consumed by humans, thus resulting in higher incidence of STEC infections during warmer months.

Because the incidence of specific non-O157 serotypes has increased, and antibiotic resistance has emerged in STEC strains associated with human infections (28, 29), we sought to determine whether antibiotic resistant STEC infections have increased in frequency in Michigan as well. Since the National Antimicrobial Resistance Monitoring System (NARMS) does not monitor resistance frequencies for the non-O157 serotypes in the US, and while increases in non-O157 infections have been observed in the US, information on antibiotic resistance in non-O157 STEC is sparse. In addition, information on factors associated with antibiotic resistant STEC infections is severely lacking. Thus, this study was undertaken to determine trends in antibiotic resistance in both STEC O157 and non-O157 isolates over a 14-year period in Michigan and identify factors associated with antibiotic resistance.

MATERIALS AND METHODS

Study population and STEC isolates

A total of 980 laboratory confirmed STEC isolates from Michigan patients from 2001-2014 were characterized for the study. Between January 1, 2001 and December 31, 2014, clinical isolates were collected as part of a sentinel surveillance system by the Michigan Department of

Community Health Bureau of Laboratories (now the Michigan Department of Health and Human Services- MDHHS) and the Enterics Research Investigational Network (ERIN) (30-32). STEC cases collected between 2001-2014 have previously been described by Tseng et al. (18) and in Chapter 2. The distribution of serotypes observed in this study is provided in Figure 3.2. All protocols used in this study were approved by the Institutional Review Boards at Michigan State University (MSU; Lansing, MI, USA; IRB #10-736SM) and the MDHHS (842-PHALAB). Isolates were grown at 37ºC in Luria-Bertani (BD Diagnostics) media under aerobic conditions for 18-20h and were stored in Luria-Bertani broth with 10% glycerol at -80°C until further testing.

Antimicrobial susceptibility testing

All STEC strains were screened for susceptibility to ampicillin (10 µg), trimethoprim-sulfamethoxazole/SXT (25 µg) and ciprofloxacin (5 µg) using the Kirby- Bauer disc diffusion test (33) on Mueller Hinton agar plates. The plates were incubated for 18-20 hours at 37ºC and the zone of clearance around each Oxoid™ antibiotic disc was measured in millimeters. The isolates were classified as being resistant or susceptible according to guidelines set by the Clinical Laboratory Standards Institute (CLSI) (34). The laboratory reference strain, *E. coli* ATCC 25922, was used as the quality control organism because it is susceptible to all antibiotics. Isolates were defined as multidrug resistant if they were resistant to all three antibiotics.

Epidemiological data

Demographic data and other epidemiological data for a subset of STEC isolates from 2001-2014 were extracted from the Michigan Disease Surveillance System (MDSS) and managed using Microsoft Excel and Access. Our prior studies (18, 32) have examined different subsets of these data, however, this study represents the first report of all case data combined over a 14-year period;

Chapter 2 examined STEC isolates collected between 2010-2014 (32), while Tseng et al. examined the epidemiology of STEC cases collected between 2001-2012 in Michigan (18). Season was classified as winter (December, January, and February), spring (March, April, and May), summer (June, July, and August) and fall (September, October, and November) based on when the case was reported; for those cases with a missing date, the date of isolation of the pathogen and referral date were used. Based on the classification scheme by the National Center for Health Statistics (NCHS), only ten Michigan counties are classified as urban (35). Moreover, antibiotic usage data in Michigan was used to classify counties as high (prescribing rates 30% higher than the state average) or low antibiotic users (36).

Clade assignments based on 32 SNP loci were included for a subset of STEC O157 isolates (n=316), which were previously characterized and recovered between 2001 and 2006 (37), to identify associations with antibiotic resistance.

Data analysis

SAS version 9.4 (SAS Institute, Cary, NC, USA) and Epi Info™ 7 were used for conducting all statistical analyses. Significant associations between variables were examined using Chi-square test and Fisher's exact test; a p value ≤ 0.05 was considered significant. The Mantel-Haenszel Chi-square test was used for analyzing trends, with a p value ≤ 0.05 considered to be significant. Those variables found to have strong associations with the dependent variable in the univariate analysis (p value ≤ 0.20) were included in the multivariate analysis to identify factors independently associated with the dependent variable. Potentially confounding factors, such as age and sex, were also included in the multivariate analysis.

RESULTS

Antimicrobial susceptibility profiles and trends in antimicrobial resistance, 2001-2014

Overall, from 2001-2014, 62 (6.3%) of the 980 STEC isolates were resistant to at least one antibiotic tested in this study. While 56 (5.7%) and 23 (2.3%) isolates were resistant to ampicillin and trimethoprim-sulfamethoxazole respectively, only one (0.1%) isolate was resistant to ciprofloxacin. Out of the 326 non-O157 isolates from 2001-2014, 32 (9.8%) were resistant to at least one antibiotic; 27 (8.3%) were resistant to ampicillin, 12 (3.7%) were resistant to trimethoprim-sulfamethoxazole and one (0.3%) isolate was resistant to ciprofloxacin. Among the 636 O157 isolates, 28 (4.4%) were resistant to at least one antibiotic, 27 (4.2%) were resistant to ampicillin, 9 (1.4%) were resistant to trimethoprim-sulfamethoxazole and all were susceptible to ciprofloxacin. A significant difference in frequencies of antibiotic resistance were observed between O157 and non-O157 (Chi square *p* value=0.001); resistance frequencies were significantly higher in non-O157 serotypes for both ampicillin and trimethoprim-sulfamethoxazole (Table 3.1). Variation in resistance frequencies were also observed when STEC strains were stratified by *stx* genes. Indeed, STEC strains with *stx1* genes were more likely to be resistant to at least one antibiotic (n=29, 9.1%) than those strains with *stx*2 alone or a combination of *stx1* and *stx*2 (n=33, 5.0%) (OR: 1.9; 95% CI: 1.13, 3.17). The same trend was observed when resistance to ampicillin and trimethoprim-sulfamethoxazole were observed; strains with *stx1* had significantly higher frequencies of resistance to ampicillin (n=25, 7.8%) and trimethoprim-sulfamethoxazole (n=12, 3.7%) than *stx*2 alone or a combination of *stx1* and *stx*2 (AMP: n=31, 4.7%; SXT: n=11, 1.7%).

Over the span of 14 years, the frequencies of antibiotic resistance $\geq$1 antibiotic in all STEC isolates significantly increased (Mantel-Haenszel *p* value=0.0074) (Table 3.2). Indeed,

significantly different frequencies in resistance were observed to trimethoprim-sulfamethoxazole (Mantel-Haenszel *p* value=0.007) and ampicillin (Mantel-Haenszel *p* value=0.028) (Table 3.2). A peak in ampicillin resistance in Michigan isolates was observed in the year 2007; however, the small sample size of 2007 isolates may be driving this trend. Although the frequency of ampicillin resistance dropped down to 5.1% in 2008 from 10.5% in 2007, this difference was not significant (Fisher's Exact *p* value=0.3). When stratified by serotype, differences in frequencies of resistance were also observed by year. While an increasing trend in trimethoprim-sulfamethoxazole resistance in O157 serotypes (Mantel-Haenszel *p* value=0.039) (Table 3.3), no significantly different trends in resistance frequencies were observed for non-O157 isolates (Table 3.4).

On comparing resistance frequencies of STEC from 2001-2009 to isolates from 2010-2014 (Figure 3.3), significantly higher frequencies of resistance were observed in 2010-2014 (n=32, 8.9%) than in 2001-2009 (n=30, 4.8%) (OR: 1.9; 95% CI: 1.16, 3.24; Chi square *p* value=0.011); particularly, the odds of trimethoprim-sulfamethoxazole resistance were significantly higher in 2010-2014 (OR: 3.3; 95% CI: 1.41, 7.99; Chi square *p* value=0.0038). While frequencies of ampicillin resistance were higher during 2010-2014 (n=27, 7.5%) than in 2001-2009 (n=29, 4.7%), this difference was not significant (Chi square *p* value=0.06).

We also sought to compare resistance frequencies between STEC O157 isolates from Michigan (n=636) and those tested by the National Antimicrobial Resistance Monitoring System (NARMS) (n=2795) (38). Ampicillin resistance was significantly higher in Michigan (n=27, 4.2%) compared to those tested by NARMS (n=74, 2.6%) (Chi square *p* value=0.02) (Figure 3.4). When stratified by year, fluctuating frequencies of resistance were observed for Michigan isolates and NARMS isolates (Figure 3.4). Notably, significantly different frequencies in trimethoprim-sulfamethoxazole resistance were observed for NARMS O157 isolates during this period (Mantel-

Haenszel *p* value=0.008) while ciprofloxacin resistance frequencies varied over the 14-year period (Mantel-Haenszel *p* value=0.053). For ampicillin, no significant trends were observed (Mantel-Haenszel *p* value=0.20). As already mentioned, significantly different trends in trimethoprim-sulfamethoxazole resistance were also observed for STEC O157 isolates from Michigan patients (Mantel-Haenszel *p* value=0.039) (Table 3.3) but not for ampicillin resistance (Mantel-Haenszel *p* value=0.32).

Epidemiological associations with antibiotic resistant STEC infections

Factors associated with antibiotic resistant STEC, collected over the span of 14 years, were identified by univariate and multivariate analyses with resistance to at least one ($\geq$1) antibiotic as the dependent variable (Table 3.6). The univariate analysis showed that non-O157 isolates had higher odds of being resistant to at least one antibiotic when compared to O157 isolates (OR: 2.4; 95% CI: 1.39-3.99). The odds of a resistant STEC isolate carrying *stx1* genes was higher than those carrying a combination of *stx1* and *stx2* (OR: 2.4; 95% CI: 1.26-4.44). No other significant univariate associations were detected. Multivariate logistic regression, controlling for age and sex, identified serotype to be a significant predictor of resistant STEC infections with non-O157 serotypes to be less likely to be resistant than O157 serotypes (OR: 2.3; 95% CI:1.36-3.96).

Table 3.7 depicts the associations between antibiotic resistance and clinical symptoms reported for each case. Higher frequencies of resistant infections were reported in those patients with abdominal pain (n=50, 6.7%) than those who did not report abdominal pain (n=7, 4.5%), although this difference was not significant (Chi square *p* value = 0.31). Similarly, patients exhibiting body ache having higher frequencies of resistant infections (n=13, 8.7%) than those without the symptom (n=44, 5.9%). In addition, patients without HUS had higher frequencies of antibiotic resistant STEC (n=57, 6.5%) than those patients with HUS (n=0, 0%), although a larger

sample size is needed to make definite conclusions about this association. Furthermore, no significant associations between case hospitalizations and antibiotic resistant infections were observed.

We also sought to identify factors associated with antibiotic resistant STEC isolates from 2001-2009 to determine differences in factors in these time periods. The univariate and multivariate analysis (Table 3.8) identified age to be independently associated with resistant STEC infections with significantly higher odds of resistant infections occurring in persons between the ages of 0-18 and >65 compared to ages 19-64 years (OR: 2.6; 95% CI: 1.13-6.10). Serotypes of isolates were not found to be associated with resistant infections in this model.

Phylogenetic associations with antibiotic resistant STEC O157, 2001-2006

The 316 STEC O157 isolates for which SNP data was previously available were classified into 25 SNP genotypes (SGs) and were grouped into eight distinct clades (37) The antibiotic susceptibility profiles for these STEC O157 isolates were used to identify associations between clades of STEC O157 and antibiotic resistance. Antibiotic resistant STEC O157 isolates were found to belong to Clade 2 (n=3, 1.8%), Clade 3 (n=1, 2.9%), Clade 4 (n=1, 33.3%), Clade 8 (n=5, 7.6%) and Clade 9 (n=1, 25.0%) (Figure 3.5). Isolates belonging to Clade 8 were found to have higher frequencies of resistance to at least one antibiotic (n=5, 7.6%) than isolates belonging to all other clades (n=6, 2.4%); this difference was close to significance (Fisher's exact p value=0.055) and a larger sample size of isolates may be required to be analyzed to make definite conclusions. Additionally, isolates belonging to Clade 8 were observed to have higher frequencies of resistance to trimethoprim-sulfamethoxazole (n=3, 4.5%) compared to isolates belonging to other clades (n=0, 0.0%) (Fisher's exact *p* value =0.009). Among Clade 8 isolates, SG-33 (n=3, 15.8%) were more likely to be resistant to at least one antibiotic compared to isolates belonging to SG-30, SG-

31 and SG-32 (n=2, 4.3%), though this difference was not significant (Fisher's Exact *p* value =0.14).

DISCUSSION

The emergence of antibiotic resistance in clinical Shiga Toxin Producing *E. coli* (STEC) has been documented in numerous studies worldwide (28, 29, 39). Additionally, the isolation of resistant STEC from animals and food sources (28, 40, 41) has raised additional concerns about the spread of resistant STEC in the environment. Although antibiotics are not recommended for the treatment of STEC (42, 43), studies have documented the empirical use of antibiotics to treat STEC O157 infections in the US. Nelson et al. observed that patients with STEC infections reported to have received antibiotics such as fluoroquinolones, trimethoprim-sulfamethoxazole and β-lactam antibiotics (44). Furthermore, it is necessary to conduct continued surveillance of antibiotic resistance in STEC due to the possibility of horizontal transfer of resistance genes to other clinically important pathogens. In this study, we have characterized antibiotic resistance in clinical STEC isolates from 2001-2014, thus giving insights into the trends and emergence of resistance to clinically relevant antibiotics over a span of 14-years in Michigan. Furthermore, while NARMS monitors antibiotic resistance of STEC O157 isolates, it does not do so for the non-O157 serotypes which have increased in incidence in the US. Thus, this study is one of the few that looks at antibiotic resistance in both STEC O157 and non-O157 serotypes and factors associated with antibiotic resistance in STEC.

Earlier studies have documented extremely low frequencies or no resistance in STEC isolates. One such study observed no resistance to 12 antimicrobial agents in STEC O157:H7 isolates collected by the Centers for Disease Control and Prevention (CDC) between 1983 and 1985 (45). Additionally, in 1988, Ratnam et al. reported antibiotic resistance in five (2.9%) STEC

O157:H7 isolates collected from US and Canada (46). Our present study found significantly lower frequencies of resistance in STEC isolates from 2001-2009 compared to isolates from 2010-2014. This increasing trend in resistance from 2001 to 2014 is alarming as it has important implications on the management of antibiotic resistant infections in Michigan. Indeed, an estimated 23 million kg of antibiotics are used every year in the US (47) and this high use in both clinical and agricultural settings is likely to play an important role in driving the frequencies of antibiotic resistance. Furthermore, resistance to ampicillin and trimethoprim-sulfamethoxazole in 2010-2014 was significantly higher than in 2001-2009. In Michigan, trimethoprim-sulfamethoxazole (Bactrim) and penicillin antibiotics (Amoxil, Pen V) were the top drugs prescribed to adults and children in 2009 (36). In addition, penicillins and sulfas are widely used in both food-producing and nonfood-producing animals (48). Thus, the efficacy of these two classes of important antimicrobial drugs is limited due to increasing frequencies of resistance. Interestingly, low frequencies of resistance to ciprofloxacin was observed in the present study. While ciprofloxacin antibiotics are widely prescribed in adults (36), low frequencies of resistance can be explained by the finding that multiple mutations are required in the *E. coli* genome to acquire clinically significant levels of resistance (49). In addition, we observed annual variations in frequencies of resistance to different antibiotics which may be explained by seasonal antibiotic usage by year (50) and turnovers of antibiotic resistant clones in the environment (51).

Our present study observed differences in antibiotic resistance by serotypes, with higher frequencies of resistance in non-O157 serotypes than in O157 serotypes. However, in Spain, similar frequencies of resistance were seen in both O157 (41%) and non-O157 isolates (41%) when resistance to 26 antimicrobial agents was tested (28). Interestingly, Schroeder *et al*. observed high frequencies of resistance to ampicillin (21%) in clinical O157 strains isolated from different

countries (29). While the reason for variation in resistance frequencies by serotypes is not known, studies have hypothesized that differences in fitness between resistant serotypes may serve as a possible explanation for this difference (52). While this hypothesis has been explored for non-typhoidal *Salmonella* (52), no studies have looked at fitness differences between resistant serotypes of STEC. Although fitness differences between the *E. coli* O104:H4 and *E. coli* O157 serotypes have been explored, with the O104:H4 serotype found to be more viable in decreased pH and sodium nitrite than O157 serotypes (53), additional studies are warranted to demonstrate fitness differences between antibiotic resistant serotypes of STEC. Additionally, although not significant, we noted differences in resistance frequencies between isolates from Michigan and those tested by NARMS. These findings warrant further studies on how different factors such as antibiotic prescription rates and use, specific evolutionary events and prevalence of antibiotic resistant STEC clones in geographically distinct regions affect antibiotic resistance (54, 55). We also observed higher odds of antibiotic resistance in strains with *stx1* gene compared to those carrying either *stx2* or a combination of *stx1* and *stx2*. However, this association can be explained by the correlation between serotypes and *stx* genes. In our dataset, non-O157 STEC are significantly more likely to carry *stx1* genes than O157 isolates (data not shown) and have higher frequencies of antibiotic resistance than O157 isolates; thus, it is likely that serotypes are driving the association between *stx* and antibiotic resistance.

Using multivariate logistic regression, we identified non-O157 serotypes collected between 2001 and 2014, to be independently associated with antibiotic resistance infections. However, serotype was not identified as a risk factor in our model using isolates only from 2001-2009. These data suggest that STEC isolates from the latter time-period between 2010-2014 (Chapter 2) are driving the associations between serotype and antibiotic resistance. Since, Tseng et al. identified

increasing incidence of non-O157 infections from 2001-2012 (18), it is likely that the emergence of antibiotic resistance in recent years in Michigan is driven by the emergence of antibiotic resistant non-O157 infections. When looking at isolates collected between 2001-2009, multivariate logistic regression identified the age groups 0-18 and ≥65 years as predictors of antibiotic resistant STEC infections. While this association has not been observed in our previous STEC study looking at isolates from 2010-2014 (Chapter 2) (32), other studies have identified age to be a significant risk factor for resistance in other pathogens (56). Interestingly, prior antibiotic use in patients has been shown to be a risk factor for antibiotic resistance (57). High antibiotic use has been documented in children and the elderly (58, 59); thus, prior antibiotic use in patients may serve as a possible explanation for the association seen in our study. In addition to age, differences in antibiotic usage patterns by gender have also been documented (60, 61). In our study, we did observe higher frequencies of antibiotic resistant STEC isolates recovered from females (6.9%) compared to males (5.5%), although this difference was not significant. Thus, further studies on differences in antibiotic prescription and consumption by gender may also shed light on differences in the prevalence of antibiotic resistant infections by gender. However, since information on prior antibiotic use is not available in the Michigan Disease Surveillance System (MDSS), additional studies are needed to test these hypothesis for STEC resistant infections.

Furthermore, our study did not observe any associations with clinical outcomes, although other studies have also documented associations between antibiotic resistant infections and severe disease outcomes (32, 62). We previously identified antibiotic resistant STEC to be independently associated with hospitalizations, a marker of severe disease outcomes (Chapter 2). Since, we did not identify any associations between hospitalizations and antibiotic resistant STEC infections

from 2001-2014, this may indicate the emergence of a virulent and resistant group of STEC in recent years in Michigan.

Previous studies have documented the association between phylogenetic lineages and antibiotic resistance in different bacterial pathogens (28, 30, 63). Indeed, Mora et al. identified STEC O157:H7 phage types PT21/28, PT23 and PT34 to have higher number of antibiotic resistant strains (28). However, our study is the first to provide insights into the association between drug resistant STEC and SNP typing based phylogenetic lineages using a large sample of STEC O157 isolates. While resistant isolates were found belonging to multiple clades, clade 8 isolates were found to have higher frequencies of antibiotic resistance compared to isolates belonging to other clades. Previously, Manning et al. reported an increase in frequency of clade 8 STEC O157 in Michigan (37). Furthermore, isolates belonging to this clade were found to be associated with severe disease outcomes such as HUS; thus, providing insights into the virulence of this clade (37). The association between clade 8 strains and antibiotic resistance has a profound impact on human health considering its importance as a pathogen. The clade 8 lineage specific acquisition of virulence and resistance genes may contribute to the overall fitness of this lineage, thus aiding in its transmission and establishment as a highly virulent lineage of STEC O157 in Michigan (64). Whole genome sequence analysis of clade 8 strains may provide insights into the linkage between antibiotic resistance and virulence genes and may shed light on how antibiotic usage may select for maintenance of virulence genes. Indeed, Zhang et al. demonstrated that prevalence of antibiotic resistance was higher in pathogenic *E. coli* than commensal strains and provided evidence for the linkage of antibiotic resistance genes and virulence genes (65).

STEC is a serious threat to public health, resulting in numerous foodborne illnesses worldwide. Continuous surveillance and identification of factors associated with antibiotic

resistance is essential to detect the emergence of antibiotic resistance and modify the use of antibiotics in Michigan. Thus, this study was undertaken to identify the emergence of antibiotic resistant STEC populations in Michigan, to enhance our understanding of the distribution of resistance profiles and assess factors associated with antibiotic resistant STEC infections to discover novel intervention strategies.

APPENDIX

Table 3.1. **Differences in frequencies of antibiotic resistance among Shiga toxin-producing *E. coli* (STEC) O157 (n=636) and non-O157 (n=326) isolates from patients in Michigan (2001-2014)**

Antibiotic	Number (%) of Resistant Isolates		OR (95% CI)	*p* value
	Non-O157	O157		
≥1 antibiotic	32 (9.8%)	27 (4.34%)	2.4 (1.39 – 3.99)	0.001
Ampicillin	27 (8.3%)	27 (4.2%)	2.0 (1.17 – 3.53)	0.01
Trimethoprim-sulfamethoxazole	12 (3.7%)	9 (1.4%)	2.7 (1.11 – 6.38)	0.02
Ciprofloxacin	1 (0.3%)	0 (0.0%)	Undefined	0.34

Table 3.2 Frequencies and trends in antibiotic resistance in all Shiga toxin-producing *E. coli* (STEC) isolates (n=980) from Michigan by year, 2001-2014. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

Year	Total	No (%) resistance			
		≥1 Antibiotic	AMP	CIP	SXT
2001	73	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2002	83	4 (4.8%)	4 (4.8%)	0 (0.0%)	0 (0.0%)
2003	55	2 (3.6%)	2 (3.6%)	0 (0.0%)	1 (1.8%)
2004	63	2 (3.3%)	2 (3.2%)	0 (0.0%)	0 (0.0%)
2005	46	3 (6.5%)	3 (6.5%)	0 (0.0%)	2 (4.3%)
2006	44	4 (9.1%)	4 (9.1%)	0 (0.0%)	2 (4.5%)
2007	19	2 (10.5%)	2 (10.5%)	0 (0.0%)	0 (0.0%)
2008	137	7 (5.1%)	7 (5.1%)	0 (0.0%)	1 (0.7%)
2009	102	6 (5.9%)	5 (4.9%)	0 (0.0%)	2 (1.9%)
2010	69	7 (10.1%)	4 (5.8%)	1 (1.4%)	4 (5.8%)
2011	47	4 (8.5%)	4 (8.5%)	0 (0.0%)	1 (2.1%)
2012	43	5 (11.6%)	4 (9.3%)	0 (0.0%)	3 (6.9%)
2013	120	12 (10.0%)	12 (10.0%)	0 (0.0%)	3 (2.5%)
2014	79	4 (5.1%)	3 (3.8%)	0 (0.0%)	4 (5.1%)
p value*		0.0074	0.026	0.60	0.007

* *p* value calculated using Mantel-Haenszel chi square for trends.

Table 3.3 Frequencies and trends in antibiotic resistance in all Shiga toxin-producing *E. coli* (STEC) O157 isolates (n=636) from Michigan by year, 2001-2014. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

Year	Total	No (%) resistance			
		≥1 Antibiotic	AMP	CIP	SXT
2001	67	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2002	74	4 (5.4%)	4 (5.4%)	0 (0.0%)	0 (0.0%)
2003	51	2 (3.9%)	2 (3.9%)	0 (0.0%)	1 (2.0%)
2004	54	1 (1.8%)	1 (1.8%)	0 (0.0%)	0 (0.0%)
2005	41	2 (4.9%)	2 (4.9%)	0 (0.0%)	1 (2.4%)
2006	35	3 (8.6%)	3 (8.6%)	0 (0.0%)	2 (5.7%)
2007	17	2 (11.8%)	2 (11.8%)	0 (0.0%)	0 (0.0%)
2008	102	4 (3.9%)	4 (3.9%)	0 (0.0%)	0 (0.0%)
2009	48	2 (4.2%)	2 (4.2%)	0 (0.0%)	0 (0.0%)
2010	29	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2011	25	1 (4.0%)	1 (4.0%)	0 (0.0%)	0 (0.0%)
2012	26	4 (15.4%)	3 (11.5%)	0 (0.0%)	2 (7.7%)
2013	36	3 (8.3%)	3 (8.3%)	0 (0.0%)	3 (8.3%)
2014	31	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
p value*		0.21	0.32	-	0.039

* *p* value calculated using Mantel-Haenszel chi square for trends.

Table 3.4 Frequencies and trends in antibiotic resistance in all Shiga toxin-producing *E. coli* (STEC) non-O157 isolates (n=326) from Michigan by year, 2001-2014. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

Year	Total	No (%) resistance			
		≥1 Antibiotic	AMP	CIP	SXT
2001	6	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2002	9	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2003	4	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2004	9	1 (11.1%)	1 (11.1%)	0 (0.0%)	0 (0.0%)
2005	5	1 (20.0%)	1 (20.0%)	0 (0.0%)	1 (20.0%)
2006	9	1 (11.1%)	1 (11.1%)	0 (0.0%)	0 (0.0%)
2007	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
2008	28	3 (10.7%)	3 (10.7%)	0 (0.0%)	1 (3.6%)
2009	46	3 (6.5%)	2 (4.3%)	0 (0.0%)	1 (2.2%)
2010	40	7 (17.5%)	4 (10.0%)	1 (2.5%)	4 (10.0%)
2011	21	3 (14.3%)	3 (14.3%)	0 (0.0%)	1 (4.8%)
2012	17	1 (5.9%)	1 (5.9%)	0 (0.0%)	1 (5.9%)
2013	84	9 (10.7%)	9 (10.7%)	0 (0.0%)	0 (0.0%)
2014	47	3 (6.4%)	2 (4.3%)	0 (0.0%)	3 (6.4%)
p value*		0.64	0.67	0.91	0.82

* *p* value calculated using Mantel-Haenszel chi square for trends.

Table 3.5. Frequency (%) of antibiotic resistance in Shiga toxin-producing *E. coli* (STEC) O157 and non-O157 isolates in Michigan, 2001-2014 (n=980). Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole

Serotype	Total*	No. (%) Resistant Infections	No. (%) AMP Resistant Infections	No. (%) CIP Resistant Infections	No. (%) SXT Resistant Infections
O157	636	28 (4.4%)	27 (4.2%)	0 (0%)	9 (1.4%)
Non-O157	326	32 (9.8%)	27 (8.3%)	1 (0.3%)	12 (3.7%)
O45	94	5 (5.3%)	4 (4.3%)	0 (0%)	2 (2.1%)
O103	97	7 (7.2%)	5 (5.1%)	0 (0%)	4 (4.1%)
O111	37	9 (24.3%)	7 (18.9%)	0 (0%)	2 (5.4%)
O26	65	3 (4.6%)	3 (4.6%)	0 (0%)	1 (1.5%)
Other	33	8 (24.2%)	8 (24.2%)	1 (3.0%)	3 (9.1%)

*Total does not add up to 980 due to missing serotypes

Table 3.6. Univariate and multivariate analysis highlighting associations between antibiotic resistance and factors in clinical Shiga toxin-producing *E. coli* (STEC) isolates in Michigan (n=980), 2001-2014

Characteristic	Total strains*	No (%) resistant	OR (95% CI) †	*p* value‡
Pathogen factors				
Serotype				
O157	636	28 (4.4%)	1.0	
Non-O157	326	32 (9.8%)	2.4 (1.39-3.99)	0.001
stx profile				
*stx*1	320	29 (9.1%)	2.4 (1.26-4.44)	0.006
*stx*2	263	17 (6.5%)	1.6 (0.81-3.31)	0.16
*stx*1*stx*2	396	16 (4.0%)	1.0	-
Demographics and other factors				
Residence				
Urban	455	24 (5.3%)	0.8 (0.44-1.31)	0.32
Rural	500	34 (6.8%)	1.0	-
Age, y				
0-18	416	30 (7.2%)	1.4 (0.79-2.39)	0.24
19-64	470	25 (5.3%)	1.0	-
≥65	86	6 (6.9%)	1.4 (0.79-2.39)	0.24
Sex				
Male	437	24 (5.5%)	1.0	-
Female	534	37 (6.9%)	1.3 (0.75-2.18)	0.36
Antimicrobial-drug prescription rates by county				
High	276	18 (6.5%)	1.1 (0.63-1.98)	0.71
Low	679	40 (5.9%)	1.0	-
Season				
Winter and Spring	263	23 (8.7%)	1.7 (0.99-2.90)	0.052
Summer and Fall	710	38 (5.3%)	1.0	-

Multivariate Logistic Regression

Characteristic	OR	95% CI	*p* value
Sex: Female	1.3	0.76-2.29	0.31
Age, y: 0-18 and ≥65	1.5	0.89-2.64	0.12
Season: Winter and Spring	1.7	0.96-2.90	0.08
Serotype: non- O157	2.3	1.36-3.96	0.0016

* Depending on the variable examined, the number of isolates does not add up to the total (n=980) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

Table 3.6 (cont'd)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables <5 in at least one cells.

£ Logistic regression was performed using forward selection while controlling for variables that yielded significant (P≤0.05) and strong (P≤0.20) associations with hospitalization in the univariate analysis. Hosmer and Lemeshow Goodness-of-Fit test (P= 0.865). All variables were tested for collinearity by analyzing the Eigen values and condition numbers.

€ Wald 95% confidence intervals (CI)

Table 3.7. Univariate analysis highlighting associations between antibiotic resistance and clinical factors in clinical Shiga toxin-producing *E. coli* (STEC) in Michigan (n=980), 2001-2014

Clinical Factors	Total strains*	No (%) ≥1 resistance	OR (95% CI) †	*p* value‡
Abdominal pain				
Yes	743	50 (6.7%)	1.5 (0.67-3.41)	0.31
No	154	7 (4.5%)	-	-
Body ache				
Yes	150	13 (8.7%)	1.5 (0.79-2.89)	0.20
No	747	44 (5.9%)	1.0	-
Diarrhea				
Yes	627	44 (7.0%)	1.5 (0.79-2.83)	0.21
No	271	13 (4.8%)	1.0	-
Bloody diarrhea				
Yes	660	41 (6.2%)	0.9 (0.51-1.68)	0.79
No	239	16 (6.7%)	1.0	-
Hemolytic Uremic Syndrome (HUS)				
Yes	17	0 (0%)	-	0.61
No	881	57 (6.5%)		
Case Hospitalization				
Yes	400	25 (6.2%)	1.0 (0.58-1.72)	1.0
No	512	32 (6.2%)	1.0	-

* Depending on the variable examined, the number of isolates does not add up to the total (n=980) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables <5 in at least one cells.

Table 3.8. Univariate and multivariate analysis highlighting associations between antibiotic resistance and clinical factors in clinical Shiga toxin-producing *E. coli* (STEC) isolates in Michigan (n=622), 2001-2009

Characteristic	Total strains*	No (%) resistant	OR (95% CI) †	*p* value‡
Pathogen factors				
Serotype				
O157	489	20 (4.1%)	1.0	0.10
Non-O157	117	9 (7.7%)	1.9 (0.86-4.41)	-
stx profile				
*stx*1	115	4 (3.5%)	0.5 (0.17-1.68)	0.30
*stx*2	188	12 (6.4%)	1.0	-
*stx*1*stx*2	319	14 (4.4%)	0.7 (0.30-1.49)	0.32
Demographics and other factors				
Residence				
Urban	302	11 (3.6%)	0.7 (0.32-1.56)	0.38
Rural	295	15 (5.1%)	1.0	-
Age, y				
0-18	262	18 (6.8%)	2.7 (1.15-6.32)	0.017
19-64	301	8 (2.7%)	1.0	-
≥65	54	3 (5.6%)	2.1 (0.55-8.39)	0.22
Sex				
Male	264	10 (3.8%)	1.0	-
Female	349	19 (5.4%)	1.5 (0.67-3.19)	0.34
Antimicrobial-drug prescription rates by county				
High	164	5 (3.0%)	0.62 (0.23-1.66)	0.33
Low	433	21 (4.8%)	1.0	-
Season				
Winter and Spring	151	9 (5.9%)	1.4 (0.63-3.18)	0.39
Summer and Fall	467	20 (4.3%)	1.0	-

Multivariate Logistic Regression

Characteristic	OR	95% CI	*p* value
Sex: Female	1.6	0.71-3.69	0.25
Age, y: 0-18 and ≥65	2.6	1.13-6.10	0.027
Serotype: non- O157	1.9	0.84-4.68	0.11

* Depending on the variable examined, the number of isolates does not add up to the total (n=622) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

Table 3.8 (cont'd)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables <5 in at least one cells.

£ Logistic regression was performed using forward selection while controlling for variables that yielded significant (P≤0.05) and strong (P≤0.20) associations with hospitalization in the univariate analysis. Hosmer and Lemeshow Goodness-of-Fit test (P= 0.9808). All variables were tested for collinearity by analyzing the Eigen values and condition numbers.

€ Wald 95% confidence intervals (CI)

Figure 3.1. Incidence of confirmed Shiga toxin-producing *E. coli* (STEC) infections reported by the FoodNet active surveillance system in the US, 1996-2017. Data adapted from FoodNet Fast (3)

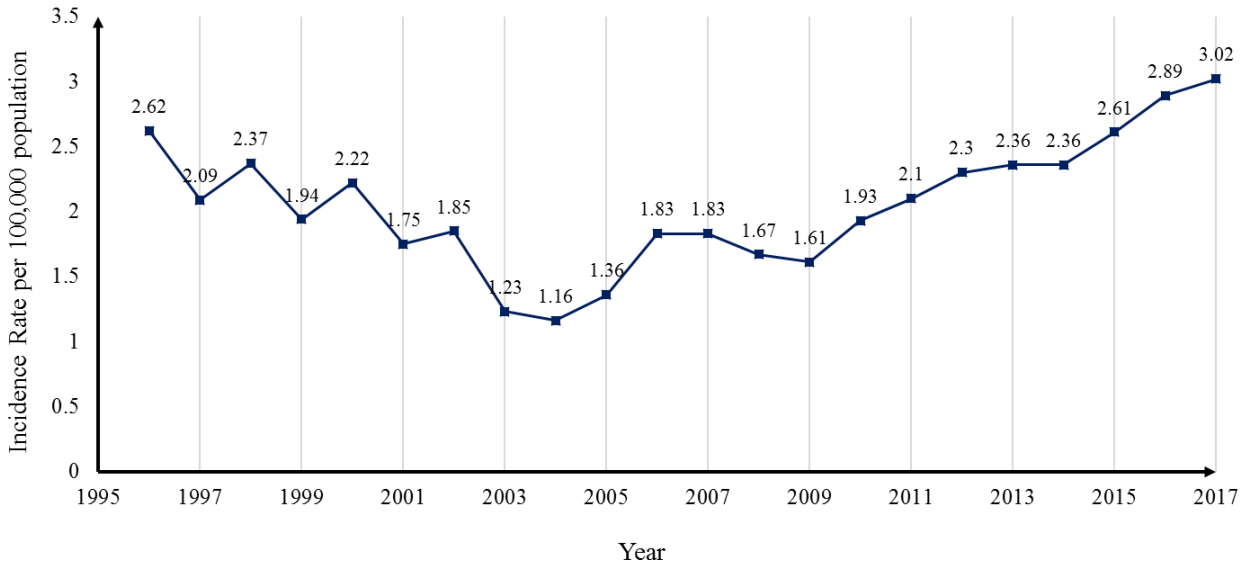


Figure 3.2. Distribution of Shiga toxin-producing *E. coli* (STEC) serotypes (n=962) in Michigan stratified by time periods, 2001-2009 (n=606) and 2010-2014 (n=356). The serotype was missing for 18 isolates and are not included in this figure

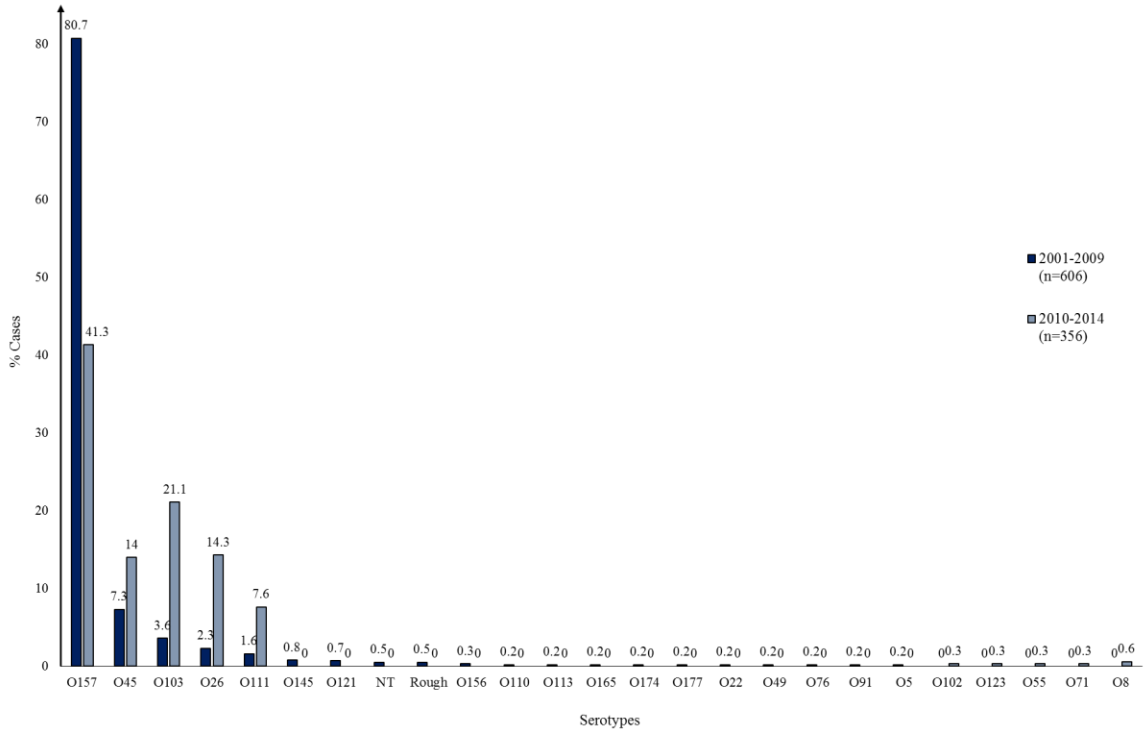


Figure 3.3. Comparison of antibiotic resistance frequencies in Shiga toxin-producing *E. coli* (STEC) isolates from 2001-2009 (n=622) and 2010-2014 (n=358). Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole (*p value≤0.05, **p value≤0.01)

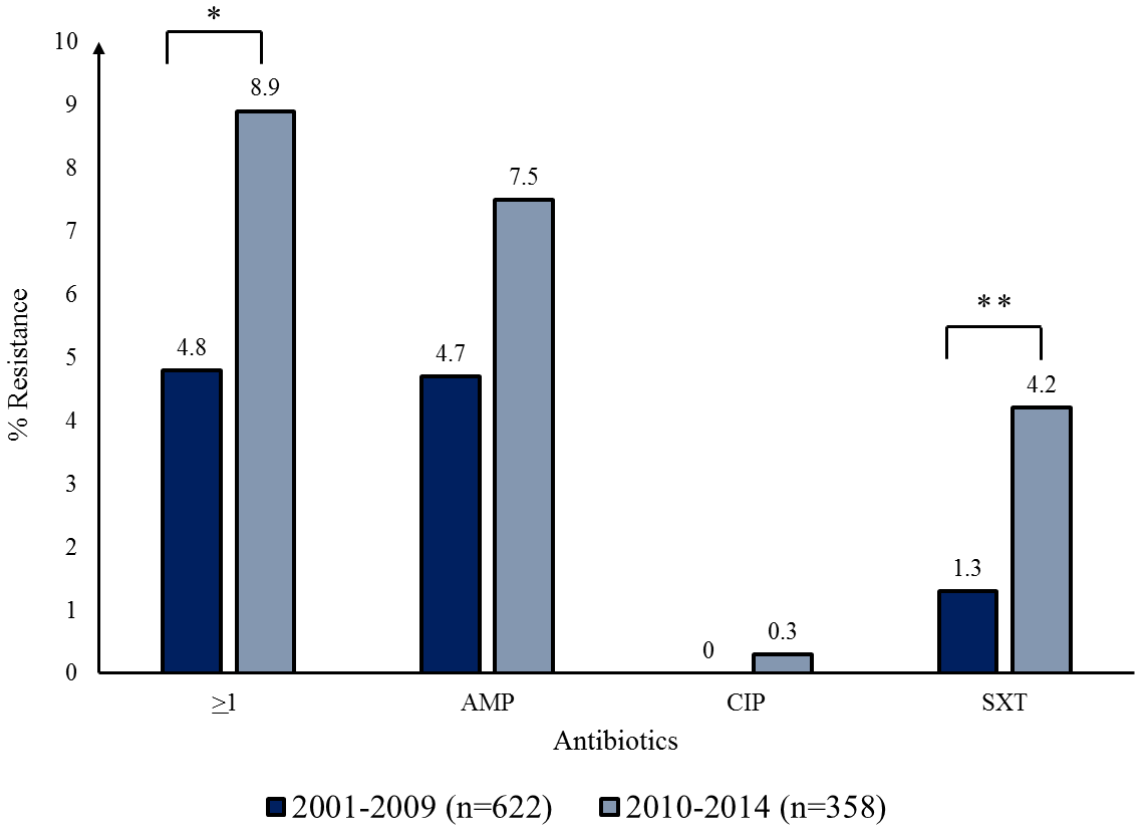


Figure 3.4. Comparison between Shiga toxin-producing *E. coli* (STEC) O157 isolates from Michigan and National Antimicrobial Resistance Monitoring System (NARMS), 2001-2014.

A) % frequency of resistance to different antimicrobial agents for the time period 2001-2014 B) % frequency of resistance to ampicillin by year, B) % frequency of resistance to ciprofloxacin by year, C) % frequency of resistance to trimethoprim-sulfamethoxazole by year. (*p value≤0.05)

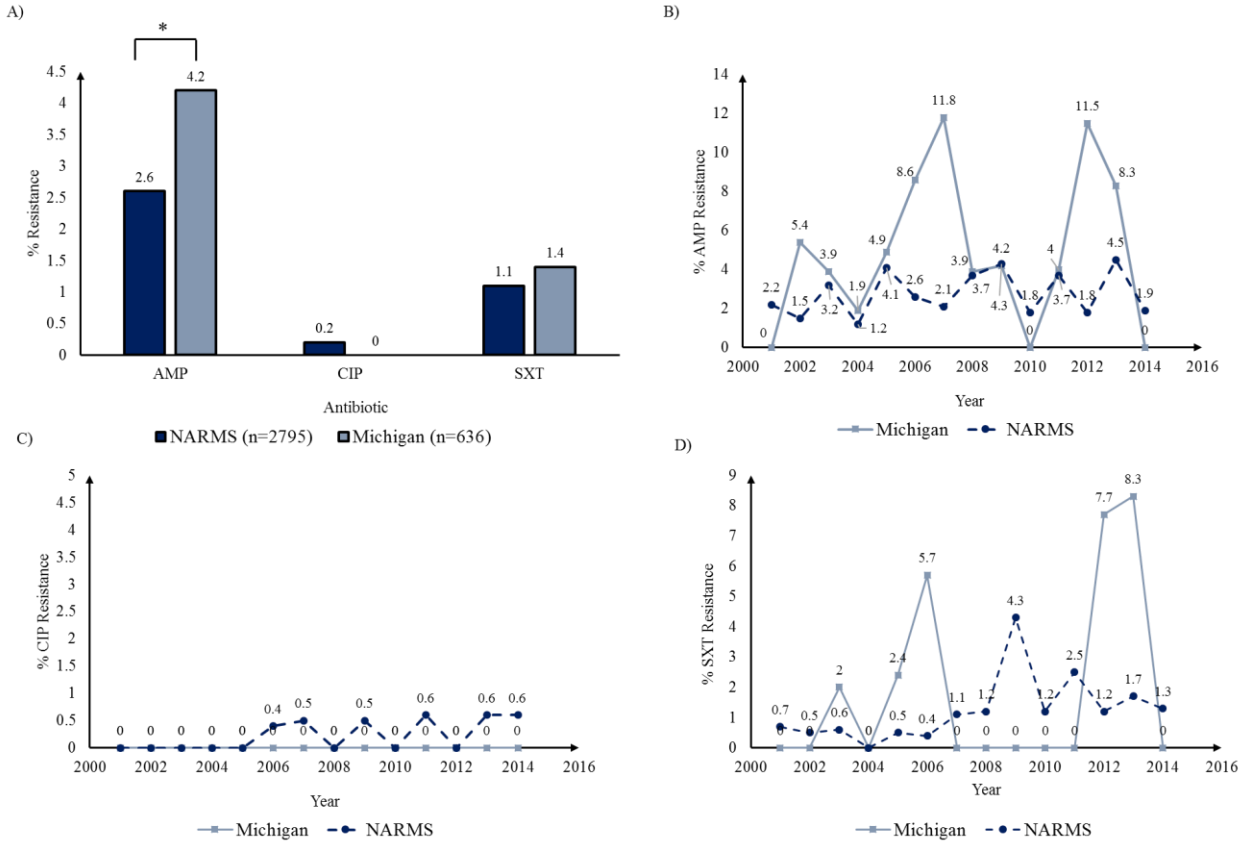
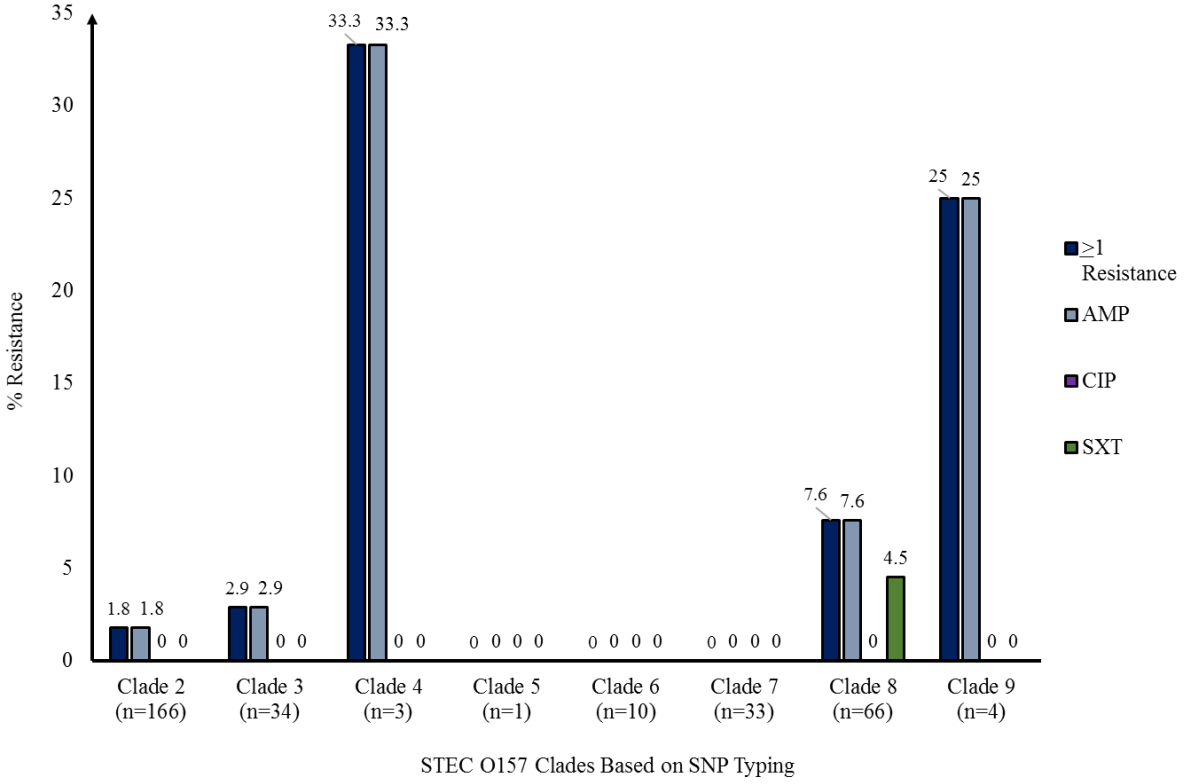


Figure 3.5. Comparison of frequencies of antibiotic resistance in Shiga toxin-producing *E. coli* (STEC) O157 (n=316) isolates from Michigan, stratified by phylogenetic clades.

Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole



REFERENCES

REFERENCES

1. Majowicz SE, Scallan E, Jones-Bitton A, Sargeant JM, Stapleton J, Angulo FJ, Yeung DH, Kirk MD. Global incidence of human Shiga toxin-producing *Escherichia coli* infections and deaths: a systematic review and knowledge synthesis. Foodborne Pathog Dis. 2014;11(6):447-55. doi: 10.1089/fpd.2013.1704.

2. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1.

3. Centers for Disease Control and Prevention. Foodborne Disease Active Surveillance Network (FoodNet): FoodNet Fast. 2017.

4. Launders N, Byrne L, Jenkins C, Harker K, Charlett A, Adak GK. Disease severity of Shiga toxin-producing *E. coli* O157 and factors influencing the development of typical haemolytic uraemic syndrome: a retrospective cohort study, 2009–2012. BMJ Open. 2016;6(1).

5. Adams NL, Byrne L, Smith GA, Elson R, Harris JP, Salmon R, Smith R, O'Brien SJ, Adak GK, Jenkins C. Shiga Toxin-Producing *Escherichia coli* O157, England and Wales, 1983-2012. Emerg Infect Diseases. 2016;22(4):590-7. doi: 10.3201/eid2204.151485.

6. Perez CE. Fruit and vegetable consumption. Health reports. 2002;13(3):23-31.

7. Marder EP, Garman KN, Ingram LA, Dunn JR. Multistate outbreak of *Escherichia coli* O157:H7 associated with bagged salad. Foodborne Pathog Dis. 2014;11(8):593-5. doi: 10.1089/fpd.2013.1726.

8. Slayton RB, Turabelidze G, Bennett SD, Schwensohn CA, Yaffee AQ, Khan F, Butler C, Trees E, Ayers TL, Davis ML, Laufer AS, Gladbach S, Williams I, Gieraltowski LB. Outbreak of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with romaine lettuce consumption, 2011. PLoS One. 2013;8(2):e55300. doi: 10.1371/journal.pone.0055300.

9. Centers for Disease Control and Prevention. *E. coli* and Food Safety. 2018.

10. Grisaru S. Management of hemolytic-uremic syndrome in children. Int J Nephrol Renovasc Dis. 2014;7:231-9. doi: 10.2147/IJNRD.S41837.

11. Scheutz F. Taxonomy Meets Public Health: The Case of Shiga Toxin-Producing *Escherichia coli*. Microbiol Spectr. 2014;2(3). doi: 10.1128/microbiolspec.EHEC-0019-2013.

12. Pierard D, Stevens D, Moriau L, Lior H, Lauwers S. Isolation and virulence factors of verocytotoxin-producing *Escherichia coli* in human stool samples. Clin Microbiol Infect. 1997;3(5):531-40.

13. Huppertz HI, Busch D, Schmidt H, Aleksic S, Karch H. Diarrhea in young children associated with *Escherichia coli* non-O157 organisms that produce Shiga-like toxin. J Pediatr. 1996;128(3):341-6.

14. Karch H, Bielaszewska M, Bitzan M, Schmidt H. Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. Diagn Microbiol Infect Dis. 1999;34(3):229-43.

15. Blanco JE, Blanco M, Alonso MP, Mora A, Dahbi G, Coira MA, Blanco J. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from human patients: prevalence in Lugo, Spain, from 1992 through 1999. J Clin Microbiol. 2004;42(1):311-9.

16. Banatvala N, Griffin PM, Greene KD, Barrett TJ, Bibb WF, Green JH, Wells JG and the Hemolytic Uremic Syndrome Study Collaborators. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. J Infect Dis. 2001;183(7):1063-70. doi: 10.1086/319269.

17. Stigi KA, Macdonald JK, Tellez-Marfin AA, Lofy KH. Laboratory practices and incidence of non-O157 shiga toxin-producing *Escherichia coli* infections. Emerg Infect Dis. 2012;18(3):477-9. doi: 10.3201/eid1803.111358.

18. Tseng M, Sha Q, Rudrik JT, Collins J, Henderson T, Funk JA, Manning SD. Increasing incidence of non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in Michigan and association with clinical illness. Epidemiol Infect. 2016;144(7):1394-405. doi: 10.1017/S0950268815002836.

19. Luna-Gierke RE, Griffin PM, Gould LH, Herman K, Bopp CA, Strockbine N, Mody RK. Outbreaks of non-O157 Shiga toxin-producing *Escherichia coli* infection: USA. Epidemiol Infect. 2014;142(11):2270-80. doi: 10.1017/S0950268813003233.

20. Brooks JT, Sowers EG, Wells JG, Greene KD, Griffin PM, Hoekstra RM, Strockbine NA. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. J Infect Dis. 2005;192(8):1422-9. doi: 10.1086/466536.

21. Centers for Disease Control and Prevention. Foodborne Diseases Active Surveillance Network (FoodNet): FoodNet 2015 Surveillance Report (Final Data). US Department of Health and Human Services, CDC. 2017.

22. Schielke A, Rosner BM, Stark K. Epidemiology of campylobacteriosis in Germany – insights from 10 years of surveillance. BMC Infect Dis. 2014;14:30-. doi: 10.1186/1471-2334-14-30.

23. Gradel K O, Dethlefsen C, C. Schonheyder H, Ejlertsen T, T. Sorensen H, Thomsen R W, Nielsen H. Severity of infection and seasonal variation of non-typhoid *Salmonella* occurrence in humans. Epidemiol Infect. 2007;135(1):93-9. doi: 10.1017/S0950268806006686.

24. Akil L, Ahmad HA, Reddy RS. Effects of Climate Change on *Salmonella* Infections. Foodborne Pathog Dis. 2014;11(12):974-80. doi: 10.1089/fpd.2014.1802.

25. Heuvelink A E, Valkenburgh S M, Tilburg J J H C, Van Heerwaarden C, Zwartkruis-Nahuis J T M, De Boer E. Public farms: hygiene and zoonotic agents. Epidemiology and Infection. 2007;135(7):1174-83. doi: 10.1017/S0950268807008072.

26. Schlager S, Lepuschitz S, Ruppitsch W, Ableitner O, Pietzka A, Neubauer S, Stöger A, Lassnig H, Mikula C, Springer B, Allerberger F. Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. Int J Med Microbiol. 2018. doi: https://doi.org/10.1016/j.ijmm.2018.06.008.

27. Venegas-Vargas C, Henderson S, Khare A, Mosci RE, Lehnert JD, Singh P, Ouellette LM, Norby B, Funk JA, Rust S, Bartlett PC, Grooms D, Manning SD. Factors Associated with Shiga Toxin-Producing *Escherichia coli* Shedding by Dairy and Beef Cattle. Appl Environ Microbiol. 2016;82(16):5049-56. doi: 10.1128/AEM.00829-16.

28. Mora A, Blanco JE, Blanco M, Alonso MP, Dhabi G, Echeita A, Gonzalez EA, Bernardez MI, Blanco J. Antimicrobial resistance of Shiga toxin (verotoxin)-producing *Escherichia coli* O157:H7 and non-O157 strains isolated from humans, cattle, sheep and food in Spain. Res Microbiol. 2005;156(7):793-806. doi: 10.1016/j.resmic.2005.03.006.

29. Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD, Meng J. Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Appl Environ Microbiol. 2002;68(2):576-81.

30. Cha W, Mosci R, Wengert SL, Singh P, Newton DW, Salimnia H, Lephart P, Khalife W, Mansfield LS, Rudrik JT, Manning SD. Antimicrobial Susceptibility Profiles of Human *Campylobacter jejuni* Isolates and Association with Phylogenetic Lineages. Front Microbiol. 2016;7:589. doi: 10.3389/fmicb.2016.00589.

31. Singh P, Teal TK, Marsh TL, Tiedje JM, Mosci R, Jernigan K, Zell A, Newton DW, Salimnia H, Lephart P, Sundin D, Khalife W, Britton RA, Rudrik JT, Manning SD. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome. 2015;3:45-. doi: 10.1186/s40168-015-0109-2.

32. Mukherjee S, Mosci RE, Anderson CM, Snyder BA, Collins J, Rudrik JT, Manning SD. Antimicrobial Drug-Resistant Shiga Toxin-Producing *Escherichia coli* Infections, Michigan, USA. Emerg Infect Dis. 2017;23(9):1609-11. doi: 10.3201/eid2309.170523.

33. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. American Society for Microbiology. 2009.

34. Clinical & Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

35. Ingram DD, Franco SJ. 2013 NCHS Urban-Rural Classification Scheme for Counties. Vital Health Stat 2. 2014(166):1-73.

36. Kofke-Egger H, Udow-Phillips M. Antibiotic Prescribing and Use. Center for Healthcare Research and Transformation. 2011.

37. Manning SD, Motiwala AS, Springman AC, Qi W, Lacher DW, Ouellette LM, Mladonicky JM, Somsel P, Rudrik JT, Dietrich SE, Zhang W, Swaminathan B, Alland D, Whittam TS. Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. Proc Natl Acad Sci U S A. 2008;105(12):4868-73. doi: 10.1073/pnas.0710834105.

38. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. 2018.

39. Momtaz H, Dehkordi FS, Hosseini MJ, Sarshar M, Heidari M. Serogroups, virulence genes and antibiotic resistance in Shiga toxin-producing *Escherichia coli* isolated from diarrheic and non-diarrheic pediatric patients in Iran. Gut Pathog. 2013;5(1):39. doi: 10.1186/1757-4749-5-39.

40. Solomakos N, Govaris A, Angelidis AS, Pournaras S, Burriel AR, Kritas SK, Papageorgiou DK. Occurrence, virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol. 2009;26(8):865-71. doi: 10.1016/j.fm.2009.06.002.

41. Amezquita-Lopez BA, Quinones B, Soto-Beltran M, Lee BG, Yambao JC, Lugo-Melchor OY, Chaidez C. Antimicrobial resistance profiles of Shiga toxin-producing *Escherichia coli* O157 and Non-O157 recovered from domestic farm animals in rural communities in Northwestern Mexico. Antimicrob Resist Infect Control. 2016;5:1. doi: 10.1186/s13756-015-0100-5.

42. Mody RK, Griffin PM. Editorial Commentary: Increasing Evidence That Certain Antibiotics Should Be Avoided for Shiga Toxin-Producing *Escherichia coli* Infections: More Data Needed. Clin Infect Dis. 2016;62(10):1259-61. doi: 10.1093/cid/ciw101.

43. Kimmitt PT, Harwood CR, Barer MR. Toxin gene expression by shiga toxin-producing *Escherichia coli*: the role of antibiotics and the bacterial SOS response. Emerg Infect Dis. 2000;6(5):458-65. doi: 10.3201/eid0605.000503.

44. Nelson JM, Griffin PM, Jones TF, Smith KE, Scallan E. Antimicrobial and antimotility agent use in persons with shiga toxin-producing *Escherichia coli* O157 infection in foodnet sites. Clin Infect Dis. 2011;52(9):1130-2.

45. Bopp CA, Greene KD, Downes FP, Sowers EG, Wells JG, Wachsmuth IK. Unusual verotoxin-producing *Escherichia coli* associated with hemorrhagic colitis. J Clin Microbiol. 1987;25(8):1486-9.

46. Ratnam S, March SB, Ahmed R, Bezanson GS, Kasatiya S. Characterization of *Escherichia coli* serotype O157:H7. J Clin Microbiol. 1988;26(10):2006-12.

47. Levy SB. Multidrug Resistance — A Sign of the Times. N Engl J Med. 1998;338(19):1376-8. doi:10.1056/NEJM199805073381909.

48. US Food and Drug Administration. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. 2011.

49. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005;41 Suppl 2:S120-6. doi: 10.1086/428052.

50. Sun L, Klein EY, Laxminarayan R. Seasonality and temporal correlation between community antibiotic use and resistance in the United States. Clin Infect Dis. 2012;55(5):687-94. doi: 10.1093/cid/cis509.

51. Montes M, Tamayo E, Mojica C, Garcia-Arenzana JM, Esnal O, Perez-Trallero E. What causes decreased erythromycin resistance in *Streptococcus pyogenes*? Dynamics of four clones in a southern European region from 2005 to 2012. J Antimicrob Chemother. 2014;69(6):1474-82. doi: 10.1093/jac/dku039.

52. Zhang CZ, Ren SQ, Chang MX, Chen PX, Ding HZ, Jiang HX. Resistance mechanisms and fitness of *Salmonella* Typhimurium and *Salmonella* Enteritidis mutants evolved under selection with ciprofloxacin in vitro. Sci Rep. 2017;7(1):9113. doi: 10.1038/s41598-017-09151-y.

53. Böhnlein C, Kabisch J, Meske D, Franz CMAP, Pichner R. Fitness of Enterohemorrhagic *Escherichia coli* (EHEC)/Enteroaggregative *E. coli* O104:H4 in Comparison to That of EHEC O157: Survival Studies in Food and *In Vitro*. Appl Environ Microbiol. 2016;82(21):6326-34. doi: 10.1128/aem.01796-16.

54. Knobler SL, Lemon SM, Najafi M, Burroughs T, editors. The Resistance Phenomenon in Microbes and Infectious Disease Vectors: Implications for Human Health and Strategies for Containment: Workshop Summary. Washington (DC): National Academies Press (US); 2003. 5, Factors Contributing to the Emergence of Resistance. Institute of Medicine (US) Forum on Emerging Infections. 2003.

55. Sannes MR, Kuskowski MA, Johnson JR. Geographical distribution of antimicrobial resistance among *Escherichia coli* causing acute uncomplicated pyelonephritis in the United States. FEMS Immunol Med Microbiol. 2004;42(2):213-8. doi: 10.1016/j.femsim.2004.05.004.

56. Faustini A, Hall AJ, Perucci CA. Risk factors for multidrug resistant tuberculosis in Europe: a systematic review. Thorax. 2006;61(2):158-63. doi: 10.1136/thx.2005.045963.

57. Pedersen G, Schonheyder HC, Steffensen FH, Sorensen HT. Risk of resistance related to antibiotic use before admission in patients with community-acquired bacteraemia. J Antimicrob Chemother. 1999;43(1):119-26.

58. Davies PDO. Does Increased Use of Antibiotics Result in Increased Antibiotic Resistance? Clin Infect Dis. 2004;39(1):18-9. doi: 10.1086/420826.

59. Malo S, Jose Rabanaque M, Feja C, Jesus Lallana M, Aguilar I, Bjerrum L. High antibiotic consumption: a characterization of heavy users in Spain. Basic Clin Pharmacol Toxicol. 2014;115(3):231-6. doi: 10.1111/bcpt.12211.

60. McGregor JC, Elman MR, Bearden DT, Smith DH. Sex- and age-specific trends in antibiotic resistance patterns of *Escherichia coli* urinary isolates from outpatients. BMC Fam Pract. 2013;14:25-. doi: 10.1186/1471-2296-14-25.

61. Schröder W, Sommer H, Gladstone BP, Foschi F, Hellman J, Evengard B, Tacconelli E. Gender differences in antibiotic prescribing in the community: a systematic review and meta-analysis. J Antimicrob Chemother. 2016;71(7):1800-6. doi: 10.1093/jac/dkw054.

62. Varma JK, Molbak K, Barrett TJ, Beebe JL, Jones TF, Rabatsky-Ehr T, Smith KE, Vugia DJ, Chang HG, Angulo FJ. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J Infect Dis. 2005;191(4):554-61. doi: 10.1086/427263.

63. Zhou Y, van den Hof S, Wang S, Pang Y, Zhao B, Xia H, Anthony R, Ou X, Li Q, Zheng Y, Song Y, Zhao Y, van Soolingen D. Association between genotype and drug resistance profiles of *Mycobacterium tuberculosis* strains circulating in China in a national drug resistance survey. PLoS ONE. 2017;12(3):e0174197. doi: 10.1371/journal.pone.0174197.

64. Reid SD, Herbelin CJ, Bumbaugh AC, Selander RK, Whittam TS. Parallel evolution of virulence in pathogenic *Escherichia coli*. Nature. 2000;406(6791):64-7. doi: 10.1038/35017546.

65. Zhang L, Levy K, Trueba G, Cevallos W, Trostle J, Foxman B, Marrs CF, Eisenberg JN. Effects of selection pressure and genetic association on the relationship between antibiotic resistance and virulence in *Escherichia coli*. Antimicrob Agents Chemother. 2015;59(11):6733-40. doi: 10.1128/AAC.01094-15.

CHAPTER 4

Characterization of Genetic Determinants of Antibiotic Resistance in Non-O157 Serotypes of Shiga Toxin-Producing *Escherichia coli* (STEC) and Association with Phylogenetic Lineage

ABSTRACT

Although the emergence of antibiotic resistance in Shiga toxin-producing *E. coli* (STEC) has been documented worldwide, there is a paucity of information about the mechanisms of antibiotic resistance and phylogenetic lineages associated with resistance in this group of pathogens. Thus, using a subset of non-O157 STEC isolates (n=208) from patients in Michigan, which were previously characterized for resistance to three antibiotics, we used whole genome sequencing (WGS) and molecular typing tools to classify the genetic determinants of resistance and identify associations with phylogenetic genetic background. WGS identified a diverse set of determinants, including chromosomal mutations and the presence of antibiotic resistance genes (ARGs). While mutations or single nucleotide polymorphisms (SNPs) in genes encoding resistance to ciprofloxacin and ampicillin were discovered among four strains, most isolates possessed horizontally acquired ARGs (n=36, 17.3%). Overall, 33 unique ARGs were detected that encoded resistance to multiple antibiotics. In addition, the *strA-strB-sul*2 genes co-occurred in 18 of the 36 isolates with horizontally acquired ARGs, thus indicating that these genes may be present on the same mobile genetic element. The proportion of resistant isolates differed significantly by multilocus sequence types (STs), with higher proportion of resistant isolates belonging to ST106 and ST119 (n=19, 82.6%) compared to other STs (n=4, 17.4%).Thus, this study is the first to provide insights into the mechanisms of antibiotic resistance in a large collection of clinical non-O157 STEC isolates in Michigan, which have been increasing in frequency since 2001. Continuous surveillance of the mechanisms of antibiotic resistance in clinical pathogens is crucial for effective treatment of patients and to develop strategies to mitigate the harmful effects of antibiotic resistance.

INTRODUCTION

The global dissemination of antibiotic resistance is a serious public health threat. A total of 23 million kilograms (kgs) of antibiotics are used every year in the US in human and veterinary medicine (1). The extensive use of antibiotics creates a strong selection pressure on bacterial populations, resulting in the transfer, selection and propagation of antibiotic resistant organisms and antibiotic resistance genes (ARGs) in humans, animals and the environment (2).

The evolution of antibiotic resistance in bacteria occurs due to mutations in existing genes or due to the acquisition of mobile genetic elements. These mutations or ARGs encode mechanisms that render the antibiotic activity of these agents ineffective against specific types of bacteria. The main mechanisms of antibiotic resistance include: i) direct modification of the antimicrobial agent, ii) alteration of drug targets or acquisition of alternative drug insensitive enzymes, iii) reduced drug permeability or use of efflux pumps (3, 4). Different resistance mechanisms are effective against different classes of antibiotics and multi-drug resistance emerges when bacteria acquire genes or mutations that encode multiple resistance mechanisms. For example, the β-lactam antibiotics, such as the penicillins, are enzymatically hydrolyzed by β-lactamases. The *bla*-$_{\text{TEM}}$ gene, which can be transferred horizontally between bacterial cells, is one such example of a β-lactamase and is prevalent worldwide (5, 6). Resistance to quinolones such as ciprofloxacin, are due to point mutations in bacterial DNA gyrase and topoisomerase IV genes that alter the target of quinolones (7), while efflux pumps are important for resistance to tetracycline, which is exported out of the bacterial cell and is encoded by *tet(A)* (8).

Numerous reports have noted the widespread prevalence of ARGs in the environment. Rodriguez-Mozaz et al. documented the presence of genes such as *bla*-$_{\text{TEM}}$, *sul*I and *qnr*S, encoding resistance or reduced susceptibility to β-lactams, sulfonamides and fluoroquinolones respectively,

in hospital effluents in Spain (9). Similar results were reported by Tao et al. in China highlighting the presence of aminoglycoside, sulfonamide and tetracycline resistance genes in pharmaceutical waste water (10). Indeed, hospital effluents are likely to be contributing to the presence of ARGs in aquatic environments (11). Other studies have documented the presence of ARGs in sewage (12) and in soil (13). Due to their widespread presence in the environment and their menacing effects, ARGs are currently considered as environmental pollutants (14, 15). In addition to the environment, the gut of humans and animals is a highly conducive environment for the horizontal transfer of genes due to the high density and diversity of microbiota (16). Metagenomic studies of the human gut resistome have identified it as a large reservoir of antibiotic resistant genes (17); for instance, Forslund et al. identified the presence of genes conferring resistance to 50 classes and subclasses in human fecal samples (18). Thus, the prevalence of ARGs in human and animal guts and their subsequent transfer between the commensal microbiota and pathogens also plays an important role in the emergence and persistence of resistance in bacterial populations.

Rapid and reliable tools are critical to identify antibiotic resistant pathogens and to control the spread and emergence of resistance. Similarly, the use of next generation sequencing has gained prominence in clinical microbiology (19) and is often used to detect the presence of antibiotic resistance genes in bacteria (20, 21). The Center for Genomic Epidemiology manages the ResFinder database, a comprehensive repository of horizontally acquired resistance genes, which enables the identification of 1,862 ARGs from 12 different antimicrobial classes (22). In addition, the PointFinder database contains information about chromosomal mutations associated with antibiotic resistance in different bacterial species (23).

The characterization of ARGs and the identification of geographical variation in the distribution of these elements is important to develop effective control strategies and policies. An

assessment of the abundance of ARGs in Michigan and identification of phylogenetic lineages associated with antibiotic resistance may help in the development of region-specific strategies to control the spread of antibiotic resistance and prevent emergence of resistance in other bacterial populations. Thus, this study was undertaken to identify the presence of ARGs in non-O157 clinical STEC isolates from Michigan and determine whether antibiotic resistance is correlated with phylogenetic lineage.

MATERIALS AND METHODS

Preparation of genomic DNA for whole genome sequencing

Bacteria were cultured overnight in Luria-Bertani (BD Diagnostics) media under aerobic conditions and harvested by centrifugation. DNA from 208 non-O157 STEC strains was extracted using the Wizard ® Genomic DNA purification kit. The samples were prepped for whole genome sequencing and sequenced using a MiSeq platform (Illumina) for 2 x 250 reads at the Research Technology Support Facility (RTSF) at MSU. Spades 3.10.1 were used to perform *de novo* genome assemblies. Trimming and quality check were conducted by Trimmomatic and FastQC, respectively. K-mers of different lengths (21, 33, 55, 77, 99, 127) were checked for quality control and were used to generate contigs that were then used for subsequent analyses.

In silico analysis of resistance genes

A comprehensive list of acquired antimicrobial resistance gene sequences were downloaded from the ResFinder3.0 database (https://cge.cbs.dtu.dk/services/ResFinder/). Due to the presence of repeats or variants of ARGs in ResFinder 3.0 database, genes with ≥85% homology were combined and interpreted as a single ARG. In-house bioinformatics scripts (https://github.com/HeatherBlankenship) were then developed to extract antibiotic resistance

genes using the Basic Local Alignment Search Tool (BLAST) (24); an E-value = 0.0001 was set to confirm specificity of the sequences extracted.

In addition to horizontally acquired ARGs, we also identified single point mutations in chromosomal genes known to contribute to antibiotic resistance. Bioinformatics scripts using the SNPpy tool (25) were used to catalog SNPs that varied between the non-O157 STEC isolates and the laboratory reference strain *E. coli* K12 strain. The web-based PointFinder tool (23) was used as the reference database for identification of SNPs; however, some genes that were not included in this database such as the *ampC* promoter and *acrR* efflux regulator were also included in this study (Table 4.1).

Multilocus sequence typing (MLST)

The EcMLST Version 1.2 database (26) (http://www.shigatox.net/ecmlst/cgi-bin/index) managed at Michigan State University was used to classify the sequence types (STs) for each isolate. The sequences of the internal fragments of the following house-keeping genes were used: *aspC* (aspartate aminotransferase), *clpX* (ATP-dependent Clp protease), *fadD* (acyl-CoA synthetase), *icdA* (isocitrate dehydrogenase), *lysP* (lysine-specific permease), *mdh* (malate dehydrogenase) and *uidA* (beta-D-glucuronidase). These seven housekeeping genes were extracted from the whole genomes using the BLAST tool via in-house bioinformatic scripts (https://github.com/HeatherBlankenship), with the E-value set as 0.0001 to ensure specificity. Gene alleles and STs were determined by submitting the sequences from these seven loci to the EcMLST database.

Data analysis

The phenotypic antibiotic resistance profiles and the presence/absence of ARGs were converted into a binary (1/0) format for statistical analysis. For phenotypic antibiotic susceptibility testing, 1 represented resistance to the respective antibiotic while 0 represented susceptibility to the respective antibiotic. For genotypic testing of antibiotic resistance, 1 and 0 represented the presence or absence of each ARG or point mutations in genes resulting in resistance, respectively. SAS software version 9.4 (SAS Institute, Cary, NC, USA), Epi Info™ 7 (27) and the open statistical program R (28) were used for all statistical analyses.

As outlined by Reller et al., the accuracy of susceptibility testing methods can be determined if the 'very major error' rate and the 'major error' rate are <1.5% and <3%, respectively (29). These rates were predicted by comparing genotypic and phenotypic resistance profiles. In this study, the 'very major error' rate was defined as isolates that were phenotypically resistant but were not genotypically resistant, or did not carry known ARGs or chromosomal gene mutations, while the 'major error' rates were defined as isolates that were phenotypically susceptible but had a resistant genotype (carried ARGs or chromosomal gene mutations) (30). The sensitivity, specificity, positive-predictive value (PPV) and negative predictive value (NPV) were calculated in SAS version 9.4.

Using a custom R script for matrix multiplication (https://github.com/Brian-No), a co-occurrence matrix of horizontally acquired ARGs was created, thus generating a count of gene occurrences.; Hence, higher values in the matrix indicates higher occurrence of two ARGs. Using the co-occurrence matrix, a network of co-occurring genes was created the open graph viz platform Gephi 0.9.2 (https://gephi.org/). The Fruchterman-Reingold algorithm was used to disperse the nodes evenly and were colored according to the degree weight.

Significant associations between antibiotic resistant infections and phylogenetic lineages and those between serotypes and phylogenetic lineages were examined using Chi-square test and Fisher's exact test; a p value ≤ 0.05 was considered significant.

RESULTS

Chromosomal mutations conferring antibiotic resistance

Overall, 13 synonymous and non-synonymous mutations were observed in the non-O157 isolates examined in this study, of which nine were non-synonymous and four were synonymous (Table 4.2). These mutations were only detected in genes conferring resistance to ciprofloxacin and ampicillin; no mutations in genes conferring resistance to tetracycline, aminoglycoside, macrolide, colistin, sulfonamide and rifamycin were detected.

Only one non-O157 strain, which was phenotypically resistant to ciprofloxacin, had multiple point mutations in *gyrA*, *parC*, *parE* and *acrR*. This isolate had a Ser-83-Leu and Asp-87-Asn amino acid change in *gyrA* (DNA gyrase subunit A), a Ser-80-Ile change in *parC* (DNA topoisomerase IV subunit A), and a Ser-458-Thr amino acid change in *parE* (DNA topoisomerase IV subunit B). Mutations in the efflux pump regulator (*acrR*) were also observed in this isolate; Thr-213-Ile and Asn-214-Thr amino acid changes observed. This same isolate was also phenotypically resistant to ampicillin and had the following SNPs: +22 (C →T), +26 (T →G), +27 (A →T) and +32(G →A), in the *ampC* promoter attenuator region (AR). This isolate was also phenotypically resistant to trimethoprim-sulfamethoxazole, however, there were no chromosomal SNPs in the *folP* gene. Two other isolates had a Ser-83-Leu amino acid change in the *gyrA* gene and one isolate had a Ser-57-Thr amino acid change in the *parC* gene, yet both isolates were phenotypically susceptible to ciprofloxacin.

Prevalence and diversity of horizontally acquired antibiotic resistance genes

Overall, 33 unique horizontally acquired ARGs were detected among all 208 non-O157 STEC genomes. The ARGs observed in this study fell in one of the three main antibiotic resistance mechanisms: antibiotic inactivation, efflux pumps and cellular protection or drug target replacement (Figure 4.1 A); most ARGs encoded antibiotic inactivating gene products (45.4%) followed by efflux pumps (39.4%) and products that resulted in protection and/or replacement of cellular targets (15.1%). When stratified by serotype, the presence of ARGs classified by mechanisms of resistance varied by STEC serotypes. However, highest proportions of genes for all three mechanisms of resistance were observed in serotypes O103 and O111 (Figures 4.1 B, C, D).

High frequencies of isolates with $\geq$1 ARG were observed; for example, 12.0% of all isolates (n=208) carried $\geq$1 aminoglycoside ARG, 10.6% carried β-lactam ARGs, 11.0% carried ARGs conferring resistance to sulfonamides, and 12.5% carried tetracycline ARGs (Figure 4.2). In addition, many isolates had more than one resistance gene for a given antibiotic. For example, only 16% (n=4) of isolates with aminoglycoside ARGs carried only one ARG, while 32% (n=8) carried two ARGs and 52% (n=13) carried $\geq$ 3 ARGs. Of the 23 isolates with sulfonamide ARGs, 52.2% (n=12), 21.7% (n=5) and 26.1% (n=6) had only one, two, and $\geq$3 ARGs, respectively. Additionally, of the 26 tetracycline ARG carrying isolates, most had only one (n=20, 76.9%) ARG while 19.2% (n=5) carried only two and 3.8% (n=1) carried $\geq$3 ARGs.

The diversity and patterns of horizontally acquired ARGs observed in STEC non-O157 isolates is depicted in Figure 4.3. For all antibiotics, except for fosfomycin and macrolides, $\geq$2 ARGs were detected.

Correlation between antibiotic resistant phenotypes and genotypes

All STEC non-O157 isolates that were phenotypically resistant (Chapter 2) to ciprofloxacin and trimethoprim-sulfamethoxazole were found to carry known ARGs or chromosomal mutations conferring antibiotic resistance to the respective antibiotics. While one isolate did have a point mutation in *gyrA* (Ser-83-Leu) and also carried the plasmid-associated gene, *qnr*, this isolate was not classified as genotypically resistant since *qnr* genes were shown to confer reduced susceptibility but not clinical levels of quinolone resistance (31). Thus, for ciprofloxacin and trimethoprim-sulfamethoxazole, the very major error and major error of genotypic antibiotic resistance profiling were calculated to be 0 (Table 4.3). Notably, one isolate that was phenotypically resistant to ampicillin did not carry any ARGs or mutations that promote resistance to penicillins. By contrast, three isolates were phenotypically susceptible to ampicillin yet were found to carry horizontally acquired ARGs such as AmpC β-lactamases (*bla*-$_{\text{CMY-2}}$ or *bla*-$_{\text{CFE-1}}$ or *bla*-$_{\text{LAT-1}}$ or *bla*-$_{\text{BIL-1}}$ or *bla*-$_{\text{ACT-1}}$ or *bla*-$_{\text{CMG}}$ or *bla*-$_{\text{MIR}}$ or their variants) and *bla*-$_{\text{TEM-1}}$ or its variants. The minimum inhibitory concentration (MIC) of these three isolates was determined using ETest® strips (bioMérieux, Inc., Durham, NC). Interestingly, while the MICs for two of these isolates were low (3µg/ml and 1.5µg/ml) and thus classified as susceptible, the third isolate was found to have an MIC of $\geq$64 µg/ml, which is resistant according to CLSI (32). Thus, there is a possibility of mis-classification of this isolate as susceptible to ampicillin using disk diffusion analysis in our previous analysis (Chapter 2). Taking this mis-classification into account, the very major error rate and major error rate for ampicillin genotypic testing were 0.48% and 0.96% respectively.

The sensitivity, specificity, PPV and NPV for genotypic determination of antibiotic resistance were also calculated (Table 4.4). For all three antibiotics, the sensitivity, specificity, PPV and NPV were >90%. For ampicillin, the PPV was the lowest (91%), which is confirmed by

the results in Table 4.3, since two isolates that were phenotypically susceptible were found to have ARGs conferring resistance to ampicillin.

Co-occurrence of horizontally acquired antibiotic resistance genes

To determine which horizontally acquired ARGs are most likely to occur together, a co-occurrence matrix was generated with a count of gene co-occurrences (Figure 4.4). Although the matrix only identified a 100 gene pairs that were detected together in the same genome more than once, a network analysis of co-occurrence patterns revealed clustering within the network (Figure 4.5). The nodes in this network represent the ARGs and the edges that connect these nodes represent connections between these nodes. Overall, 32 nodes were identified in the network; the *tet(Q)* gene, which did not have any co-occurrences with other ARGs, was excluded from the network. As indicated by the solid red edges, the ARGs that had the strongest connections with each other were *strA*, *strB* and *sul1*; indeed, these genes co-occurred in 18 (8.6%) of all STEC isolates tested. The number of isolates with co-occurrence of *strA*, *strB*, and *sul2* were significantly higher than the following gene combinations: *sul2-aac(3)-IIa* (0.5%, Chi-square *p* value<0.0001), *sul2-bla*-$_{CMY2}$/*bla*-$_{CFE1}$/*bla*-$_{LAT1}$/*bla*-$_{BIL1}$ (1.9%, Chi-square *p* value<0.003), *sul2-mphA* (1.9%, Chi-square *p* value<0.003) and *sul2-dfrA12* (1.9%, Chi-square *p* value<0.003). In addition, *sul2* co-occurred with *tet(A)* as 13 (6.2%) of the 208 non-O157 isolates had both genes, while *tet(A)* was also found with *strA* (n=13, 6.2%), *strB* (n=13, 6.2%) and *aph(3)-Ia* (n=13, 6.2%). Although *strA-strB-sul2* co-occurred together more frequently than other ARG combinations, *sul1*, *sul3* and *aadA1* displayed the most connections to other ARGs in the network. Specifically, *sul1* and *sul2* were connected to 24 genes and *aadA1* co-occurred with 25 other ARGs.

Associations between non-O157 sequence types (STs) and antibiotic resistance

A total of 10 different STs, including one new ST, were identified among the 208 non-O157 STEC isolates examined in this study (Figure 4.6); the ST of two isolates could not be classified due to sequencing errors and were excluded from this analysis. In all, the most prevalent ST was ST119 (n=119, 57.8%) followed by ST106 (n=77, 37.4%); six STs were represented by one isolate each (Figure 4.6). When stratified by ST and serotype (Table 4.5), 46 O45 (97.8%) and 71 O103 (95.9%) isolates grouped into ST119 while most O26 (n=48, 94.1%) and O111 (n=26, 96.3%) isolates belonged to ST106.

On examining the relationship between antibiotic resistance patterns and STs, isolates representing ST106 (n=10, 12.9%) were found to have highest frequencies of resistance to at least one antibiotic followed by ST119 (n=9, 7.6%) (Figure 4.7); this difference was, however, not significant (Chi square *p* value=0.2). Isolates belonging to ST106 (n=8, 10.4%) also had higher frequencies of resistance to ampicillin than ST119 isolates (n=7, 5.9%), though not significant (Chi square *p* value=0.2). Several STs such as ST 288, ST86, ST104 and ST171 had only one resistant isolate each. The isolate belonging to ST-288 was multidrug resistant as it was resistant to all three classes of antibiotics tested in this study. Of the 23 antibiotic resistant isolates examined here, the proportion of antibiotic resistant STs differed significantly by STs; higher proportions of resistant isolates belonged to STs 106 and 119 (n=19, 82.6%) compared to other STs (n=4, 17.4%) (*p* value=0.0018). Of all the serotypes examined in this study, the highest frequency of antibiotic resistant isolates belonged to serotype O111 (n=6, 22.2%) and were significantly more likely than other non-O157 serotypes (n=17, 9.4%) to be resistant to at least one antibiotic (Chi square *p* value=0.048). Furthermore, serotype O111 were significantly more likely than other serotypes to belong to ST106 (Fisher's Exact Chi square *p* value<0.0001). When stratified by both serotype

and STs, all resistant O111 isolates belonged to ST106 (n=6, 23.1%) (Table 4.6). These findings suggest the certain serotypes and lineages may be more likely to be resistant. However, a larger sample size of isolates belonging to other STs other than ST119 and ST106 may need to be analyzed to fully discern differences between antibiotic resistant isolates belonging to different STs and serotypes.

DISCUSSION

Current trends in increasing antibiotic resistance warrant the need for rapid identification of antibiotic resistance. In addition, surveillance of antibiotic resistance has an important impact on designing policies and strategies to control the spread of antibiotic resistance. The affordability and rapidity of whole genome sequencing (WGS) has made it an attractive tool for screening of antibiotic resistance (33, 34). Thus, many studies have characterized antibiotic resistance using WGS technology. For instance, McDermott et al. used WGS to characterize antibiotic resistance in 640 non-typhoidal *Salmonella* and reported the correlation between phenotypic and genotypic testing in 99% of the cases (35). The use of WGS for the prediction of antibiotic resistance has also been employed in other pathogens such as *Staphylococcus aureus* (36), *Enterococcus faecalis* (20) and *Campylobacter* (37).

However, the use of WGS in clinical decision making is hampered by the lack of published studies using WGS to predict antibiotic testing of bacteria. Indeed, the European Committee on Antimicrobial Susceptibility (EUCAST) highlights the lack of published evidence in different bacteria as a major barrier to using WGS for antibiotic resistance detection in clinical settings (38). In addition, EUCAST calls for international standardization and quality control (QC) metrics for interpretation of WGS based antibiotic resistance detection (38). Hence, a goal of this study was

to determine the potential of WGS to be used for the detection of antimicrobial resistance in STEC non-O157 isolates and identify ARGs prevalent in Michigan.

We observed a high concordance between the results from phenotypic antimicrobial testing using disc diffusion and prediction of resistance using WGS. For ciprofloxacin, the sensitivity and specificity of the genomic prediction method were 1.0 (95% CI: 0.025-1.0) and 1.0 (95% CI: 0.98-1.0), respectively, and the very major error and major error rate were also found to be 0. It is important to note, however, that since the frequencies of phenotypic ciprofloxacin resistance was low (n=1, 0.48%), a larger sample size may be required to accurately determine the PPV and NPV of genotypic ciprofloxacin testing (39). The sensitivity (1.0, 95% CI: 0.69-1.0) and specificity (1.0, 95% CI: 0.98-1.0) of genetically predicting trimethoprim-sulfamethoxazole resistance was also high with very major and major error rates of 0. For ampicillin, however, there were three instances of disagreement, thus resulting in lower sensitivity (0.95, 95% CI: 0.76-0.99) and specificity (0.98, 95% CI: 0.96-0.99) values. One isolate that was classified as phenotypically resistant to ampicillin was negative for the presence of known ARGs linked to ampicillin resistance. The sequencing coverage for this isolate was adequate (25.23X), thereby eliminating the possibility that ampicillin resistance genes could not be detected due to poor sequencing coverage. This observation highlights an important limitation of using WGS to identify antimicrobial resistance; this tool only identifies known ARGs and mechanisms of antibiotic resistance and would prevent the identification of novel genes and mechanisms. Since we only used the ResFinder 3.0 database, it is possible that inclusion of additional databases such as the Comprehensive Antibiotic Resistance Database (CARD) or Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) may aid in the identification of novel ARGs that are not included in the ResFinder 3.0 database. Future work will involve using CARD and ARG-ANNOT databases to identify the genes responsible for ampicillin

resistance in the phenotypically resistant but genotypically susceptible isolate. The ResFinder 3.0 database was chosen as the reference database in this study because both the National Antimicrobial Resistance Monitoring System (NARMS) and FDA utilize it for WGS based detection of ARGs in non-typhoidal *Salmonella* (40, 41). While the Resistome Tracker has been launched by the FDA to provide information about ARGs present in in non-typhoidal *Salmonella* (41), there is a dearth in information about ARGs in STEC considering that the CDC does not consider antibiotic resistant STEC to be a serious threat to public health, yet (42). However, considering the ease with which ARGs can be transmitted, it is important to identify the emergence of resistant genes in different regions, to control the spread of resistant infections.

Similarly, two isolates carrying *bla* genes, which were previously found to be phenotypically susceptible in our previous study (Chapter 2), were re-tested using ampicillin Etest® strips; both isolates had MICs <3µg/ml, confirming susceptibility to ampicillin. Few studies have documented *bla-$_{TEM}$* positive and ampicillin sensitive strains. For instance, two *bla-$_{TEM}$* positive, ampicillin-sensitive strains of *Haemophilus influenzae* were found to produce lower β-lactamase levels due to the presence a mutation in the *bla*-$_{\text{TEM}}$ promoter or due to an amino acid substitution rendering the *bla*-$_{\text{TEM}}$ enzyme inactive (43). Furthermore, MICs have been shown to be dependent on the type of promoter expressing *bla*-$_{\text{TEM}}$ genes. In *E. coli* transformed with *bla-$_{TEM\text{-}1B}$* gene, for instance, the MICs of antibiotics such as amoxicillin-clavulanate, ticarcillin-clavulanate, piperacillin, and cephalothin varied depending on the type of promoter used (44). Similarly, high level expression of plasmid-associated *ampC* β-lactamases such as *bla-$_{ACT}$* and *bla-$_{MIR}$*, also depend on the promoter (45); thus, further characterization of these phenotypically susceptible isolates is needed. Although WGS analysis was found to be consistent with phenotypic identification of clinically resistant non-O157 isolates, it is important to note that a well-curated

database is crucial for high concordance between phenotypic and genotypic susceptibility testing, in turn improving the sensitivity of genotypic testing.

Although three isolates had single point mutations in *parC* or *gyrA*, which typically impact fluoroquinolone resistance (7), these isolates were not phenotypically resistant to ciprofloxacin. In gram negative bacteria, resistance mutations resulting in amino acid substitutions first occur in the 'quinolone resistance determining region' (QRDR) of *gyrA*. The first mutation results in reduced susceptibility to quinolones, while further mutations in other genes such as *parC* or *gyrA* are required to achieve clinical levels of resistance (7). In addition to point mutations, plasmid mediated resistance to quinolones was also observed in two isolates. The *qnrA* and *qnrS* genes, which encode pentapeptides that protect DNA gyrase and topoisomerase IV from the effect of quinolones (46), and the *qepA* gene, encoding an efflux pump (46, 47) were identified. Because these genes have been shown to result in small increases in the MICs of quinolones (46), they are not likely to confer clinical levels of resistance to ciprofloxacin. These findings highlight the importance of the concomitant use of genotypic and phenotypic antibiotic susceptibility testing, especially in clinical settings, to prevent the reporting of false-positives or false-negatives, which in turn could affect patient treatment and care.

We have also provided evidence of co-occurring horizontally acquired ARGs, confirming relationships that have been described in other studies (30, 48). Three genes, *strA-strB-sul2*, were observed to have the highest counts of co-occurrence. A prior study of atypical enteropathogenic *E. coli* (aEPEC) isolated from children in South Asia and sub-Saharan-Africa also found *sul2*, *strA* and *strB* to co-occur along with *bla*-$_{\text{TEM}}$ (30). This result may be explained by the finding that commensal *E. coli* strains can carry a *strA*, *strB* and *sul2* resistance gene cassette on pCERC1, a small 6.8kb plasmid (49). This cassette has also been found in the RSF1010 plasmid along with

others (50-52). In addition, the co-occurrence of *strA-strB-sul2-tet(A)* was observed in 13 isolates in our study. A variant of the p9123 plasmid containing the *tet(A)* gene next to the inverted repeats (IR) of the Tn5393 transposon containing the *strA-strB-sul2* cassette has been reported (49) as have small plasmids (pSS046) carrying all four resistance genes in *Shigella* isolates from the UK (53). Considering the ease of transmission of horizontally transferrable elements and the importance of multidrug resistance as the efficacy of antibiotics that can be used for treatment of infections is limited, the co-selection of multiple resistance genes is a major public health concern. Not only are the lack of antibiotic drug development programs to combat multidrug resistance a huge concern (54), but also results in high morbidity and mortality (2, 55, 56). While many genes were found to co-occur in this study, the *tet(Q*) gene was not found to co-occur with any other genes. This gene, which is found on a conjugative transposon, has mostly been reported in *Bacteroides (57).* The finding of this gene in an STEC genome highlights the importance of horizontal gene transfer between different bacterial genera and the need for continuous surveillance for ARGs to track the emergence of resistance to antibiotics.

We observed significantly higher proportions of antibiotic resistant STEC belonging to ST106 and ST119. Furthermore, all resistant isolates belonging to ST 106 and ST119, were serotypes O111 and O45 respectively. While our findings may indicate that antibiotic resistance is prevalent in certain genetic backgrounds or serotypes of STEC isolates in Michigan, a larger sample size of isolates belonging to STs other than ST106 and ST119 may be required to be tested to make definite conclusions. The distribution of ARGs conferring resistance to ampicillin and trimethoprim-sulfamethoxazole on different branches of the phylogenetic tree indicates the widespread distribution of these genes in different lineages and serotypes of STEC. Since most ARGs were identified as those transmitted by mobile genetic elements, the ease with which these

genes are transmitted among bacteria belonging to the same or different genera is of great concern from a public health perspective.

While our study is one of the few that looks at genetic determinants of antibiotic resistance in STEC non-O157 isolates, it has a few limitations. The minimum sequencing depth recommended by EUCAST for the use of WGS for antibiotic resistance testing is 30X. In our study, the depth of sequencing varied between 18X and 65X; with 43.7% (n=91) of the genomes had a coverage greater than or equal to 30X, 53.4% (n=111) had a coverage between 20X and 30X and 6 (2.9%) had genomic coverage less than 20. Two of the three isolates with discordant phenotypic and genotypic ampicillin susceptibility results had a coverage between 20X and 30X, while one had a high coverage of 63.5X.

The presence of horizontally transferred ARGs in STEC is of great significance due to the possibility of transfer to other STEC or other pathogenic organisms. Additionally, knowledge about the different mechanisms of resistance conferring resistance or reduced susceptibility to antibiotics is crucial to determine changing patterns of antibiotic resistance in Michigan.

APPENDIX

Table 4.1. List of chromosomal genes examined for point mutations conferring antibiotic resistance

Antibiotic	Genes	Reference
Ciprofloxacin	*gyrA*	(23, 58, 59)
	gyrB	(23, 58, 60)
	parC	(23, 58, 61)
	parE	(23, 58)
	acrR	(7, 62)
Tetracycline	16S *rrsB*	(23, 63)
Ampicillin	*ampC* promoter	(64-66)
Aminoglycosides		
• Gentamicin	*16S rrsB*	(23)
• Kasugamycin	*16S rrsC*	(23)
• Spectinomycin	*rrsH*	(23)
	rrsB	
Macrolide	23S *rRNA*	(23, 67)
Colistin	*pmrA*	(23, 68)
	pmrB	(23, 68)
Sulfonamide	*folP*	(23)
Rifamycin	*rpoB*	(23, 69)

Table 4.2. Synonymous and non-synonymous mutations in known resistance genes among Shiga toxin-producing *E. coli* (STEC) non-O157 isolates relative to *E. coli* ATCC 25922.

Abbreviation: S, Synonymous; NS- Non-Synonymous

Gene	Nucleotide position relative to *E. coli* K12	Isolate Number				
		TW18931	TW18499	TW18567	TW18574	TW19061
parE	A1317G	S* (remains Pro)	-	-	-	-
	T1372G	-	NS (Ser to Thr)	-	-	-
parC	G239T	-	NS (Ser to Ile)	-	-	-
	C168T	-	-	-	S (remains Ala)	-
	G170C	-	-	-	NS (Ser to Thr)	-
gyrA	C248T	-	NS (Ser to Leu)	NS (Ser to Leu)	-	NS (Ser to Leu)
	C255T	-	S (remains Val)	-	-	-
	G259A	-	NS (Asp to Asn)	-	-	-
acrR	T86G	-	S* (remains Leu)	-	-	-
	C638T	-	NS (Thr to Ile)	-	-	-
	A641C	-	NS (Asn to Thr)	-	-	-
ampC promoter	C+22T	-	Yes*	-	-	-
	T+26G	-	Yes*	-	-	-
	A+27T	-	Yes*	-	-	-
	G+32A	-	Yes*	-	-	-

Table 4.2 (cont'd)

* less than <15 reads

Table 4.3. Correlation between phenotypic and genotypic antimicrobial resistance profiles of Shiga toxin-producing *E. coli* (STEC) non-O157 isolates (n=208)

Antibiotic	No. with Resistant Phenotype	No. with Resistant Genotype and Resistant Phenotype	Very Major Error£	No. with Susceptible Phenotype	No. with Susceptible Genotype and Susceptible Phenotype	Major Error€
Ampicillin	21	20	1 (0.48%)	187	185	2 (0.96%)
Ciprofloxacin*	1	1	0	208	208	0
Trimethoprim-sulfamethoxazole†	10	10	0	198	198	0

£ Very major error occurs if a phenotypically resistant isolate is genotyped as susceptible. In other words, this error is the failure to detect phenotypic resistance using genotypic methods.

€ Major error occurs if a phenotypically susceptible isolate is genotyped as resistant. In other words, the genotypic tests predict there is resistance when there is none.

* Genotypic resistance to ciprofloxacin is defined by the presence of at least one SNP in *gyrA* and a second in *gyrA*, *gyrB*, *parC* or *parE*. The presence of plasmid mediated determinants such as *qnr* or *qepA*, plus the first SNP in *gyrA* does not confer clinical resistance, but reduced susceptibility, to quinolones.

† Genotypic resistance to trimethoprim-sulfamethoxazole is defined as the presence of both a *dfr* gene and a *sul* gene.

Table 4.4. Predictive power of antibiotic resistant genotypes for antibiotic resistant phenotypes

Antibiotic	Sensitivity (95% CI*)	Specificity (95% CI*)	PPV (95% CI*)	NPV (95% CI*)
Ampicillin	0.95 (0.76-0.99)	0.98 (0.96-0.99)	0.91 (0.71-0.99)	0.99 (0.97-0.99)
Ciprofloxacin	1.0 (0.025-1.0)	1.0 (0.98-1.0)	1.0 (0.025-1.0)	1.0 (0.98-1.0)
Trimethoprim-sulfamethoxazole	1.0 (0.69-1.0)	1.0 (0.98-1.0)	1.0 (0.69-1.0)	1.0 (0.98-1.0)

*Exact Confidence Interval

Table 4.5. Distribution of serotype profiles of non-O157 Shiga toxin-producing *E. coli* (STEC) (n=205) by MLST sequence type (ST) in Michigan, 2010-2014

Sequence Types (ST)	Number (%) strains*				
	O45	O103	O26	O111	Other
ST119	46 (97.8%)	71 (95.9%)	1 (1.9%)	1 (3.7%)	0 (0%)
ST106	0 (0%)	2 (2.7%)	48 (94.1%)	26 (96.3%)	1 (16.7%)
ST288	0 (0%)	0 (0.0%)	0 (0%)	0 (0%)	1 (16.7%)
ST86	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (16.7%)
ST104	0 (0%)	0 (0%)	1 (1.9%)	0 (0%)	1 (16.7%)
ST1062	1 (2.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
ST145	0 (0%)	0 (0%)	1 (1.9%)	0 (0%)	0 (0%)
ST73	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (16.7%)
ST171	0 (0%)	1 (1.3%)	0 (0%)	0 (0%)	0 (0%)
New ST	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (16.7%)
Total Isolates	47	74	51	27	6

*The total number of strains does not add up to 208 since two O45 isolates had missing sequence type and the serotype of one isolate was unknown; thus, these isolates were excluded from the table.

Table 4.6. Univariate analysis identifying sequence types (STs), stratified by serotype, associated with antibiotic resistance in non-O157 Shiga toxin-producing *E. coli* (STEC) in Michigan 2010-2014

Variable	Total strains*	Univariate analysis		
		No (%) Resistant	Odds Ratio (95% CI) †	*p* value‡
Serotype O111	27	6 (22.2%)		
ST119	1	0 (0.0%)	1.0	-
ST106	26	6 (23.1%)	Undefined	1.0
Other STs	0	-	-	-
Serotype O45	47	5 (10.6%)		
ST119	46	5 (10.9%)	Undefined	1.0
ST106	0	-	-	-
Other STs	1	0 (0.0%)	1.0	-
Serotype O103	74	5 (6.8%)		
ST119	71	4 (5.6%)	0.0 (Undefined)	0.07
ST106	2	0 (0.0%)	0.0 (Undefined)	0.33
Other	1	1 (100.0%)	1.0	-
Serotype O26	51	3 (5.9%)		
ST119	1	0 (0.0%)	0.0 (Undefined)	0.33
ST106	48	3 (6.2%)	Undefined	1.0
Other	2	0 (0.0%)	1.0	-
Other serotypes	6	4 (66.7%)		
ST119	0	-	-	-
ST106	1	1 (100.0%)	0.0 (Undefined)	1.0
Other	5	3 (60.0%)	1.0	-

*Depending on the variable examined, the number of isolates does not add up to the total (n=209) because of missing data. In addition, isolates of a certain serotype may not belong to certain STs and are indicated by a zero value.

† 95% confidence interval (CI) for odds ratio (OR)

Table 4.6 (cont'd)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables ≤5 in at least one cells. € Wald 95% confidence intervals (CI)

Figure 4.1. Distribution of horizontally acquired antibiotic resistance genes (ARGs) (n=33) by mechanism of action. A) Proportion of genes by mechanism of resistance; B) Proportion of antibiotic inactivation genes present in STEC genomes by serotypes (n=30); C) Proportion of antibiotic efflux genes present in STEC genomes by serotypes (n=24); and D) Proportion of cellular protection/target replacement genes present in STEC genomes by serotypes (n=24)

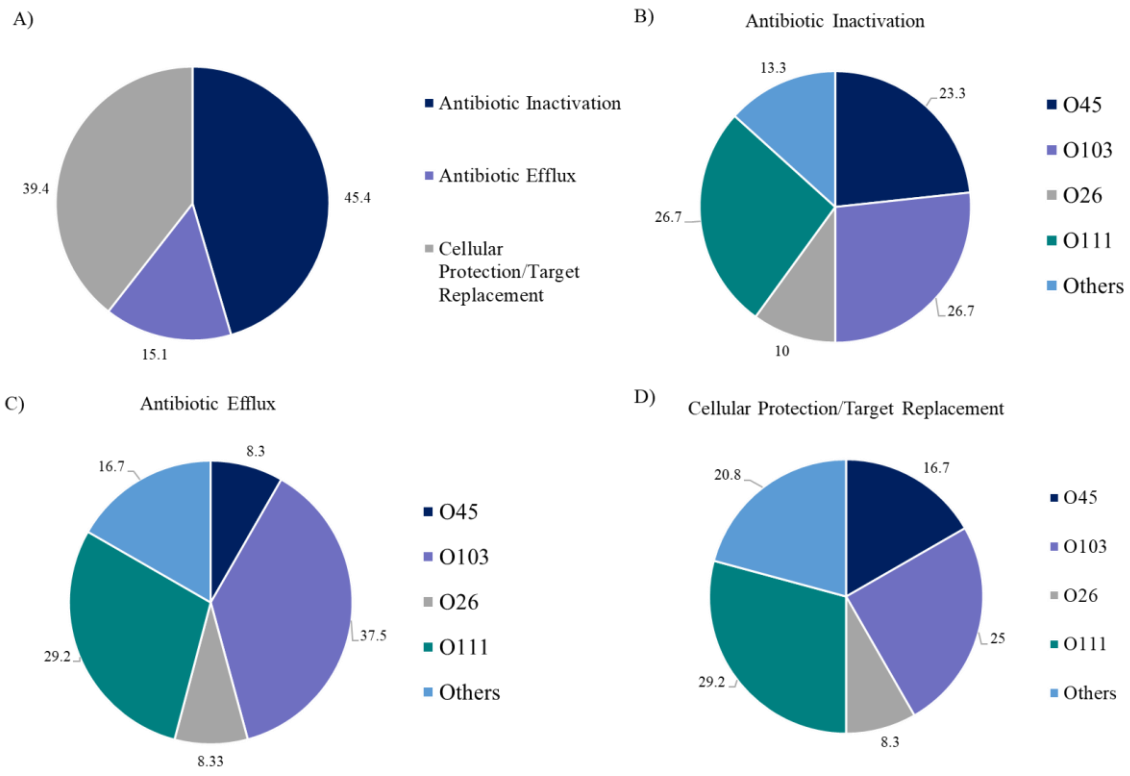


Figure 4.2. Frequencies of Shiga toxin-producing *E. coli* (STEC) non-O157 isolates (n=208) with at least one horizontally acquired antibiotic resistance gene

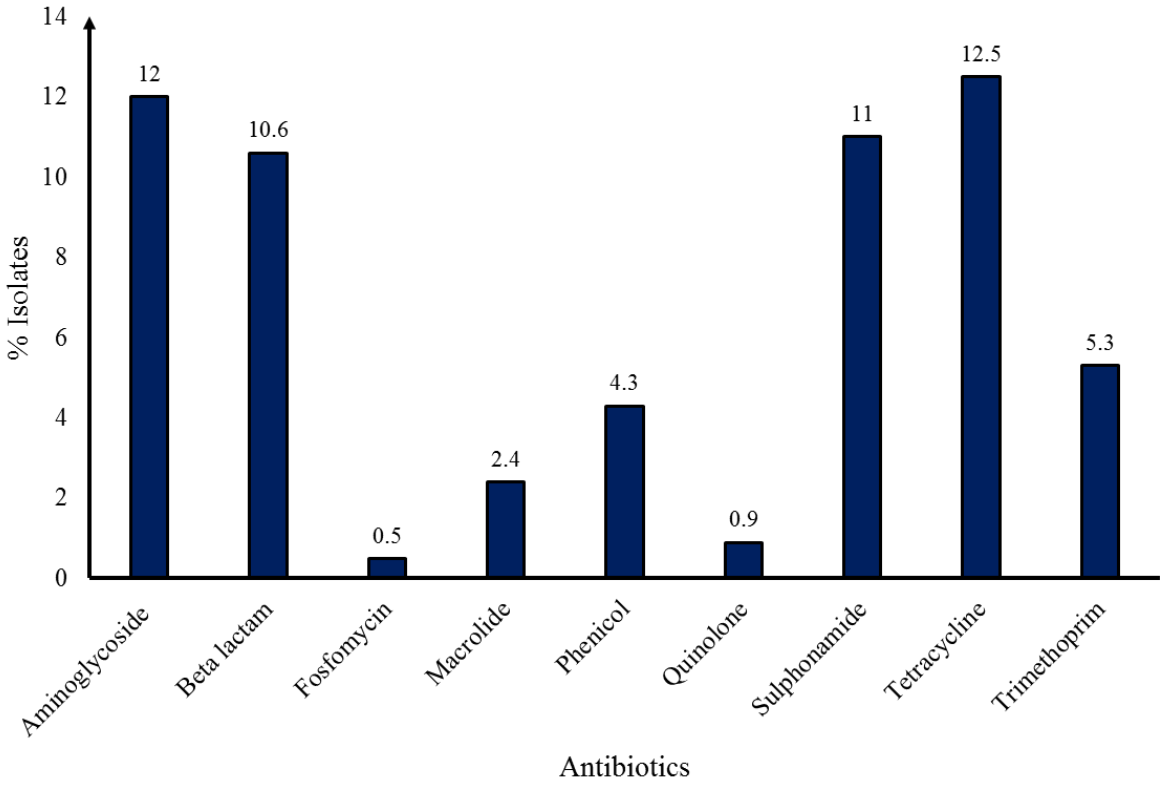


Figure 4.3. Diversity of horizontally acquired antibiotic resistance genes (ARGs) detected in 208 Shiga toxin-producing *E. coli* (STEC) non-O157 isolates in Michigan

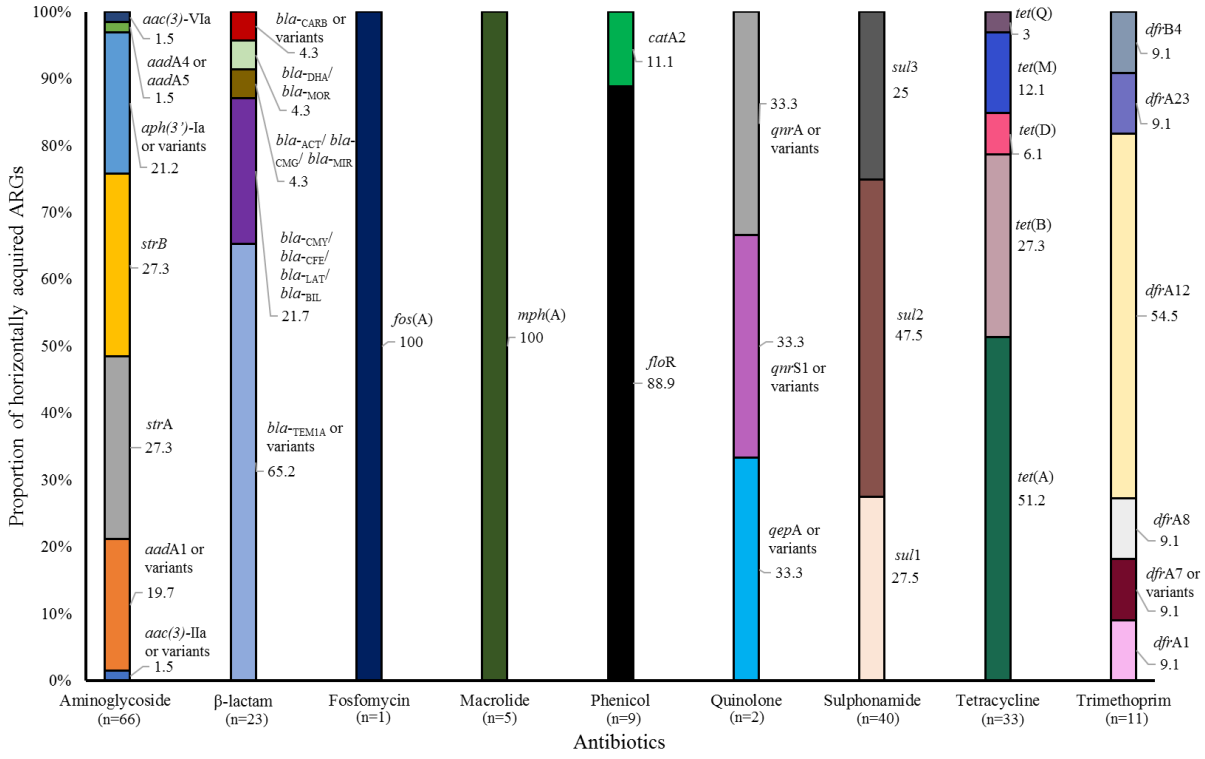


Figure 4.4. Co-occurrence matrix of horizontally acquired antibiotic resistance genes (ARGs). The matrix represents the co-occurrence gene counts. Larger values are represented by a darker shade of green and indicate higher co-occurrence of two ARGs

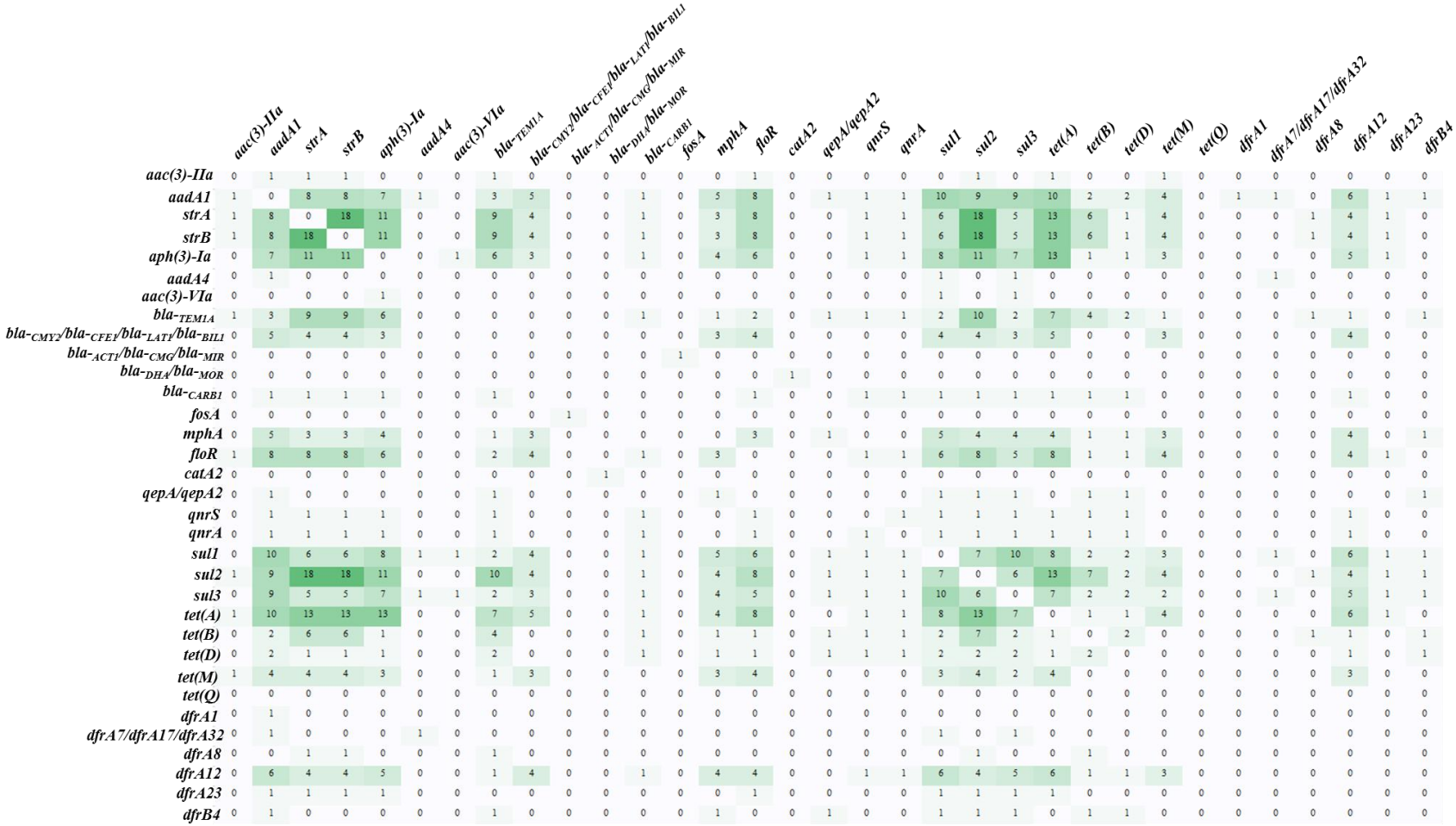


Figure 4.5. Network analysis depicting co-occurrence of horizontally acquired ARGs in 208 Shiga toxin-producing *E. coli* (STEC) non-O157 isolates. Larger and dark blue nodes indicate that these ARGs have more connections than smaller, red colored nodes. Additionally, the solid red edge lines connecting ARG nodes indicate that these ARGs occur together more frequently than those nodes connected by dashed blue lines

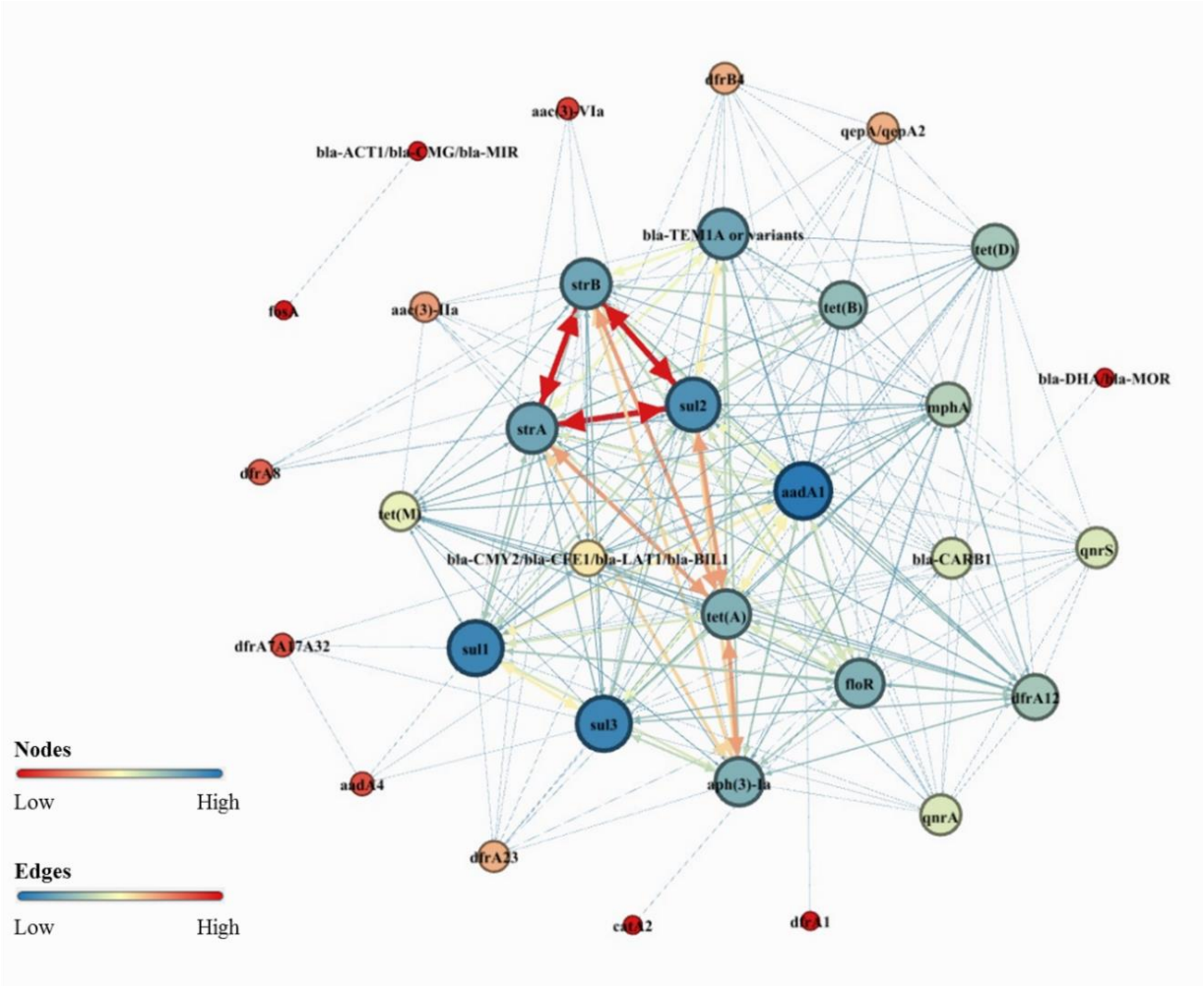


Figure 4.6. Distribution of Shiga toxin-producing *E. coli* (STEC) non-O157 sequence types (STs) in Michigan, 2010-2014

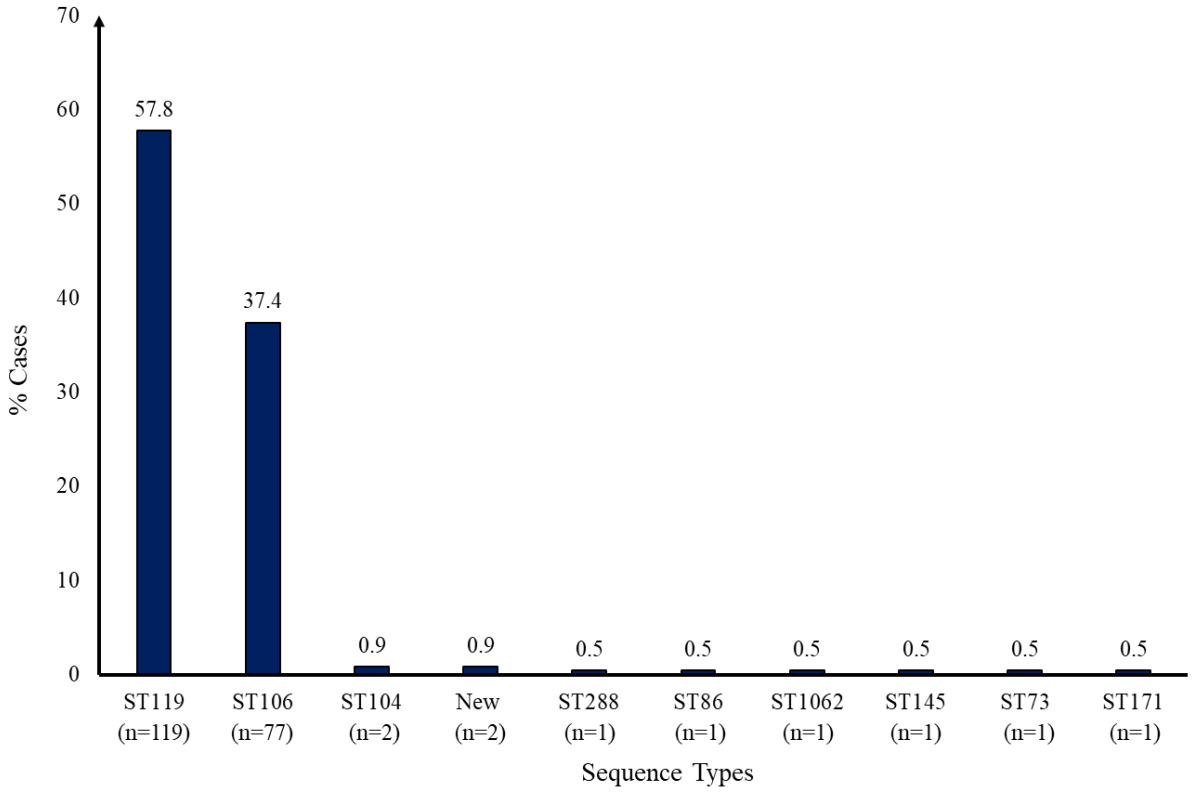
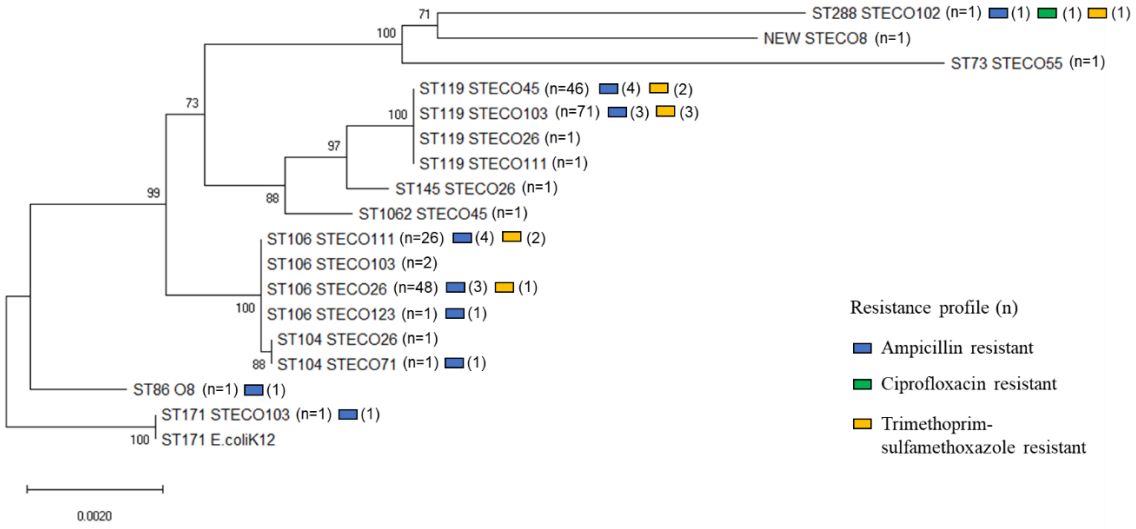


Figure 4.7. Evolutionary relationship between non-O157 Shiga toxin-producing *E. coli* (STEC) (n=205) from 2011-2014 by MLST sequence types defined by seven loci and antimicrobial resistance patterns. The evolutionary relationship was inferred by the Neighbor-Joining method; and the evolutionary distances were computed using the Maximum Composite Likelihood model and pairwise deletion of positions with gaps. The Bootstrap values (1000 replicates) are indicated next to the branches which represent the % of replicates that support the branch. MEGAX software was used to generate the tree



REFERENCES

REFERENCES

1. Levy SB. Multidrug Resistance — A Sign of the Times. N Eng J Med. 1998;338(19):1376-8. doi:10.1056/NEJM199805073381909.

2. Ventola CL. The Antibiotic Resistance Crisis: Part 1: Causes and Threats. Pharm Ther. 2015;40(4):277-83.

3. Munita JM, Arias CA. Mechanisms of Antibiotic Resistance. Microbiol Spectr. 2016;4(2):10. doi: 10.1128/microbiolspec.VMBF-0016-2015.

4. Blair JMA, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJV. Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol. 2014;13:42. doi: 10.1038/nrmicro3380.

5. Bradford PA. Extended-Spectrum β-Lactamases in the 21st Century: Characterization, Epidemiology, and Detection of This Important Resistance Threat. Clin Microbiol Rev. 2001;14(4):933-51. doi: 10.1128/CMR.14.4.933-951.2001.

6. Samaha-Kfoury JN, Araj GF. Recent developments in β lactamases and extended spectrum β lactamases. BMJ : Br Med J. 2003;327(7425):1209-13.

7. Jacoby GA. Mechanisms of resistance to quinolones. Clin Infect Dis. 2005;41 Suppl 2:S120-6. doi: 10.1086/428052.

8. Chopra I, Roberts M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol Mol Biol Rev. 2001;65(2):232-60. doi: 10.1128/MMBR.65.2.232-260.2001.

9. Rodriguez-Mozaz S, Chamorro S, Marti E, Huerta B, Gros M, Sànchez-Melsió A, Borrego CM, Barceló D, Balcázar JL. Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river. Water Res. 2015;69:234-42. doi: https://doi.org/10.1016/j.watres.2014.11.021.

10. Tao W, Zhang XX, Zhao F, Huang K, Ma H, Wang Z, Ye L, Ren H. High Levels of Antibiotic Resistance Genes and Their Correlations with Bacterial Community and Mobile Genetic Elements in Pharmaceutical Wastewater Treatment Bioreactors. PLoS One. 2016;11(6):e0156854. doi: 10.1371/journal.pone.0156854.

11. Rowe WPM, Baker-Austin C, Verner-Jeffreys DW, Ryan JJ, Micallef C, Maskell DJ, Pearce GP. Overexpression of antibiotic resistance genes in hospital effluents over time. J Antimicrob Chemother. 2017;72(6):1617-23. doi: 10.1093/jac/dkx017.

12. Su JQ, An XL, Li B, Chen QL, Gillings MR, Chen H, Zhang T, Zhu YG. Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China. Microbiome. 2017;5(1):84. doi: 10.1186/s40168-017-0298-y.

13. Peng S, Feng Y, Wang Y, Guo X, Chu H, Lin X. Prevalence of antibiotic resistance genes in soils after continually applied with different manure for 30 years. J Hazard Mater. 2017;340:16-25. doi: 10.1016/j.jhazmat.2017.06.059.

14. Martinez JL. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ Pollut. 2009;157(11):2893-902. doi: https://doi.org/10.1016/j.envpol.2009.05.051.

15. Pruden A, Pei R, Storteboom H, Carlson KH. Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. Environ Sci Technol. 2006;40(23):7445-50.

16. Lerner A, Matthias T, Aminov R. Potential Effects of Horizontal Gene Exchange in the Human Gut. Front Immunol. 2017;8:1630-. doi: 10.3389/fimmu.2017.01630.

17. van Schaik W. The human gut resistome. Philos Trans R Soc Lond B Biol Sci. 2015;370(1670):20140087-. doi: 10.1098/rstb.2014.0087.

18. Forslund K, Sunagawa S, Kultima JR, Mende DR, Arumugam M, Typas A, Bork P. Country-specific antibiotic use practices impact the human gut resistome. Genome research. 2013;23(7):1163-9. doi: 10.1101/gr.155465.113.

19. Didelot X, Bowden R, Wilson DJ, Peto TEA, Crook DW. Transforming clinical microbiology with bacterial genome sequencing. Nature Reviews Genetics. 2012;13:601. doi: 10.1038/nrg3226.

20. Zankari E, Hasman H, Kaas RS, Seyfarth AM, Agerso Y, Lund O, Larsen MV, Aarestrup FM. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. J Antimicrob Chemother. 2013;68(4):771-7. doi: 10.1093/jac/dks496.

21. Stoesser N, Batty EM, Eyre DW, Morgan M, Wyllie DH, Del Ojo Elias C, Johnson JR, Walker AS, Peto TE, Crook DW. Predicting antimicrobial susceptibilities for *Escherichia coli* and *Klebsiella pneumoniae* isolates using whole genomic sequence data. J Antimicrob Chemother. 2013;68(10):2234-44. doi: 10.1093/jac/dkt180.

22. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67(11):2640-4. doi: 10.1093/jac/dks261.

23. Zankari E, Allesøe R, Joensen KG, Cavaco LM, Lund O, Aarestrup FM. PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with

chromosomal point mutations in bacterial pathogens. J Antimicrob Chemother. 2017;72(10):2764-8. doi: 10.1093/jac/dkx217.

24. McGinnis S, Madden TL. BLAST: at the core of a powerful and diverse set of sequence analysis tools. Nucleic Acids Res. 2004;32(suppl_2):W20-W5. doi: 10.1093/nar/gkh435.

25. Mitha F, Herodotou H, Borisov N, Jiang C, Yoder J, Owzar K. SNPpy--database management for SNP data from genome wide association studies. PLoS One. 2011;6(10):e24982. doi: 10.1371/journal.pone.0024982.

26. Weihong Q, Lacher DW, Bumbaugh AC, Hyma KE, Ouellette LM, Large TM, Tarr CL, Whittam TS, editors. EcMLST: an online database for multi locus sequence typing of pathogenic *Escherichia coli*. Proceedings 2004 IEEE Computational Systems Bioinformatics Conference, 2004 CSB 2004; 2004 19-19 Aug. 2004.

27. Centers for Disease Control and Prevention. Epi Info™. 2017.

28. R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2018.

29. Reller LB, Weinstein M, Jorgensen JH, Ferraro MJ. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. Clin Infect Dis. 2009;49(11):1749-55. doi: 10.1086/647952.

30. Ingle DJ, Levine MM, Kotloff KL, Holt KE, Robins-Browne RM. Dynamics of antimicrobial resistance in intestinal *Escherichia coli* from children in community settings in South Asia and sub-Saharan Africa. Nat Microbiol. 2018;3(9):1063-73. doi: 10.1038/s41564-018-0217-4.

31. Redgrave LS, Sutton SB, Webber MA, Piddock LJV. Fluoroquinolone resistance: mechanisms, impact on bacteria, and role in evolutionary success. Trends Microbiol. 2014;22(8):438-45. doi: 10.1016/j.tim.2014.04.007.

32. Clinical & Laboratory Standards Insitute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

33. Köser CU, Ellington MJ, Peacock SJ. Whole-genome sequencing to control antimicrobial resistance. Trends Genet. 2014;30(9):401-7. doi: 10.1016/j.tig.2014.07.003.

34. Schurch AC, van Schaik W. Challenges and opportunities for whole-genome sequencing-based surveillance of antibiotic resistance. Ann N Y Acad Sci. 2017;1388(1):108-20. Epub 2017/01/31. doi: 10.1111/nyas.13310.

35. McDermott PF, Tyson GH, Kabera C, Chen Y, Li C, Folster JP, Ayers SL, Lam C, Tate HP, Zhao S. Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal *Salmonella.* Antimicrob Agents Chemother. 2016;60(9):5515.

36. Gordon NC, Price JR, Cole K, Everitt R, Morgan M, Finney J, Kearns AM, Pichon B, Young B, Wilson DJ, Llewelyn MJ, Paul J, Peto TEA, Crook DW, Walker AS, Golubchik T. Prediction of *Staphylococcus aureus* Antimicrobial Resistance by Whole-Genome Sequencing. J Clin Microbiol. 2014;52(4):1182-1191; doi: 10.1128/JCM.03117-13.

37. Zhao S, Tyson GH, Chen Y, Li C, Mukherjee S, Young S, Lam C, Folster JP, Whichard JM, McDermott PF. Whole-Genome Sequencing Analysis Accurately Predicts Antimicrobial Resistance Phenotypes in *Campylobacter* spp. Appl Environ Microbiol. 2016;82(2):459-66. doi: 10.1128/aem.02873-15.

38. Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, Grundman H, Hasman H, Holden MTG, Hopkins KL, Iredell J, Kahlmeter G, Köser CU, MacGowan A, Mevius D, Mulvey M, Naas T, Peto T, Rolain JM, Samuelsen Ø, Woodford N. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect. 2017;23(1):2-22. doi: https://doi.org/10.1016/j.cmi.2016.11.012.

39. Steinberg DM, Fine J, Chappell R. Sample size for positive and negative predictive value in diagnostic research using case–control designs. Biostatistics. 2009;10(1):94-105. doi: 10.1093/biostatistics/kxn018.

40. National Antimicrobial Resistance Monitoring System. NARMS: 2015 Human Isolates Surveillance Report. 2015.

41. U.S. Food and Drug Administration. Global Salmonella Resistome Data. 2018.

42. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

43. Tristram SG. Novel blaTEM-positive ampicillin-susceptible strains of *Haemophilus influenzae*. J Infect Chemother. 2009;15(5):340-2. doi: https://doi.org/10.1007/s10156-009-0700-1.

44. Lartigue MF, Leflon-Guibout V, Poirel L, Nordmann P, Nicolas-Chanoine M-H. Promoters P3, Pa/Pb, P4, and P5 Upstream from bla(TEM) Genes and Their Relationship to β-Lactam Resistance. Antimicrob Agents Chemother. 2002;46(12):4035-7. doi: 10.1128/AAC.46.12.4035-4037.2002.

45. Reisbig MD, Hanson ND. Promoter sequences necessary for high-level expression of the plasmid-associated ampC beta-lactamase gene blaMIR-1. Antimicrob Agents Chemother. 2004;48(11):4177-82. doi: 10.1128/AAC.48.11.4177-4182.2004.

46. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. Plasmid-mediated quinolone resistance: a multifaceted threat. Clin Microbiol Rev. 2009;22(4):664-89. doi: 10.1128/cmr.00016-09.

47. Yamane K, Wachino J-i, Suzuki S, Kimura K, Shibata N, Kato H, Shibayama K, Konda T, Arakawa Y. New Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, Found in an *Escherichia coli* Clinical Isolate. Antimicrob Agents Chemother. 2007;51(9):3354-60. doi: 10.1128/AAC.00339-07.

48. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. BMC Genomics. 2015;16:964. doi: 10.1186/s12864-015-2153-5.

49. Anantham S, Hall RM. pCERC1, a small, globally disseminated plasmid carrying the *dfrA14* cassette in the *strA* gene of the *sul2-strA-strB* gene cluster. Microb Drug Resist. 2012;18(4):364-71. doi: 10.1089/mdr.2012.0008.

50. Yau S, Liu X, Djordjevic SP, Hall RM. RSF1010-like plasmids in Australian *Salmonella enterica* serovar Typhimurium and origin of their *sul2-strA-strB* antibiotic resistance gene cluster. Microb Drug Resist. 2010;16(4):249-52. doi: 10.1089/mdr.2010.0033.

51. Scholz P, Haring V, Wittmann-Liebold B, Ashman K, Bagdasarian M, Scherzinger E. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene. 1989;75(2):271-88.

52. Liu X. Characterisation of antibiotic resistance gene clusters and their mobility within a collection of multi-drug resistant *Salmonella* spp. University of Wollongong Australia. 2018. University of Wollongong Thesis Collection 1954-2016: 3043

53. Baker KS, Dallman TJ, Field N, Childs T, Mitchell H, Day M, Weill F-X, Lefèvre S, Tourdjman M, Hughes G, Jenkins C, Thomson N. Genomic epidemiology of *Shigella* in the United Kingdom shows transmission of pathogen sublineages and determinants of antimicrobial resistance. Sci Rep. 2018;8(1):7389. doi: 10.1038/s41598-018-25764-3.

54. Freire-Moran L, Aronsson B, Manz C, Gyssens IC, So AD, Monnet DL, Cars O. Critical shortage of new antibiotics in development against multidrug-resistant bacteria—Time to react is now. Drug Resist Updat. 2011;14(2):118-24. doi: https://doi.org/10.1016/j.drup.2011.02.003.

55. Mathur P, Singh S. Multidrug resistance in bacteria: a serious patient safety challenge for India. J Lab Physicians. 2013;5(1):5-10. doi: 10.4103/0974-2727.115898.

56. van Duin D, Paterson DL. Multidrug-Resistant Bacteria in the Community: Trends and Lessons Learned. Infect Dis Clin North Am. 2016;30(2):377-90. doi: 10.1016/j.idc.2016.02.004.

57. Speer BS, Shoemaker NB, Salyers AA. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin Microbiol Rev. 1992;5(4):387-99.

58. Ruiz J. Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. J Antimicrob Chemother. 2003;51(5):1109-17. doi: 10.1093/jac/dkg222.

59. Saenz Y, Zarazaga M, Brinas L, Ruiz-Larrea F, Torres C. Mutations in *gyrA* and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products, humans and animals. J Antimicrob Chemother. 2003;51(4):1001-5. doi: 10.1093/jac/dkg168.

60. Yoshida H, Bogaki M, Nakamura M, Yamanaka LM, Nakamura S. Quinolone resistance-determining region in the DNA gyrase *gyrB* gene of *Escherichia coli*. Antimicrob Agents Chemother. 1991;35(8):1647-50.

61. Vila J, Ruiz J, Goñi P, De Anta MT. Detection of mutations in *parC* in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrob Agents Chemother. 1996;40(2):491-3.

62. Wang H, Dzink-Fox JL, Chen M, Levy SB. Genetic Characterization of Highly Fluoroquinolone-Resistant Clinical *Escherichia coli* Strains from China: Role of *acrR* Mutations. Antimicrob Agents Chemother. 2001;45(5):1515-21. doi: 10.1128/AAC.45.5.1515-1521.2001.

63. Ross JI, Eady EA, Cove JH, Cunliffe WJ. 16S *rRNA* Mutation Associated with Tetracycline Resistance in a Gram-Positive Bacterium. Antimicrob Agents Chemother. 1998;42(7):1702-5.

64. Tracz DM, Boyd DA, Bryden L, Hizon R, Giercke S, Van Caeseele P, Mulvey MR. Increase in *ampC* promoter strength due to mutations and deletion of the attenuator in a clinical isolate of cefoxitin-resistant *Escherichia coli* as determined by RT-PCR. J Antimicrob Chemother. 2005;55(5):768-72. doi: 10.1093/jac/dki074.

65. Caroff N, Espaze E, Gautreau D, Richet H, Reynaud A. Analysis of the effects of -42 and -32 ampC promoter mutations in clinical isolates of *Escherichia coli* hyperproducing *ampC*. J Antimicrob Chemother. 2000;45(6):783-8.

66. Yu W, Bing L, Zhenhua L. AmpC promoter and attenuator mutations affect function of three *Escherichia coli* strains. Curr Microbiol. 2009;59(3):244-7. doi: 10.1007/s00284-009-9426-7.

67. Ettayebi M, Prasad SM, Morgan EA. Chloramphenicol-erythromycin resistance mutations in a 23S *rRNA* gene of *Escherichia coli*. J Bacteriol. 1985;162(2):551-7.

68. Quesada A, Porrero MC, Tellez S, Palomo G, Garcia M, Dominguez L. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. J Antimicrob Chemother. 2015;70(1):71-4. doi: 10.1093/jac/dku320.

69. Jin DJ, Gross CA. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. J Mol Biol. 1988;202(1):45-58.

CHAPTER 5

Antimicrobial Resistance Profiles of Shiga Toxin-Producing *Escherichia coli* (STEC) recovered from Cattle in Michigan

ABSTRACT

The importance of cattle as a reservoir of Shiga toxin-producing *E. coli* (STEC) has been reported in numerous studies, with STEC prevalence rates per herd reported to be up to 70%. In addition, high frequencies of resistance to antibiotics important in both human and veterinary medicine have been reported in STEC isolates from animals. The primary goal of this study was determining the frequency of antibiotic resistance in STEC isolates recovered from cattle in Michigan. A total of 121 STEC isolates were recovered from 75 fecal samples in cattle at six farms in Michigan; isolates were tested for susceptibility to ampicillin, ciprofloxacin, trimethoprim-sulfamethoxazole and tetracycline. Non-O157 isolates had significantly higher frequencies of antibiotic resistance (50.0%) than O157 isolates (0.0%) (Fisher's exact *p* value<0.0001). All resistant isolates were observed to be *eae*-negative and isolates possessing *stx2* (55.9%) more likely to be resistant than those with *stx1* only or both *stx1* and *stx2* (4.8%) [Odds Ratio (OR): 24.9; 95% Confidence Interval (CI): 7.0, 88.8). At the animal level, 28% of the 75 animals had at least one antibiotic resistant isolate, with high frequencies of tetracycline (28%) resistance and trimethoprim-sulfamethoxazole resistance (16%); resistance to ampicillin and ciprofloxacin were not observed. Resistant STEC was only detected in beef farms (42.0%) compared to dairy farms (0.0%) (Fisher's exact *p* value<0.0001) and resistance to trimethoprim-sulfamethoxazole was only observed in one beef herd. Two antibiogram patterns were observed; nine (12%) isolates were resistant to tetracycline and 12 (16.0%) were resistant to both tetracycline and trimethoprim-sulfamethoxazole. Continuous monitoring of antibiotic resistance in clinical as well as animal STEC isolates is warranted to help combat the emerging problem of antibiotic resistance in STEC. In addition, identification of farm and animal management specific factors may also play a crucial role in controlling the spread of antibiotic resistance.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC), an important human pathogen, is commonly isolated from livestock such as cattle, sheep and pigs (1-3). The role of cattle as a reservoir has gained attention due to the high prevalence rates of both O157 and non-O157 serotypes (3-6) and the number of outbreaks linked to beef products (7). Moreover, occupational and recreational contact with animals, including cattle, were identified as important risk factors for STEC infection in humans (8-10). These findings have prompted the study of factors associated with STEC shedding in cattle (11-13); for instance, Venegas-Vargas et al. identified temperature and lactation period to be important for STEC shedding in dairy cattle (11). The identification of such factors is crucial to develop long-term strategies to reduce shedding levels in reservoir animals and reduce the likelihood of STEC transmission to humans.

Antibiotics are widely used in food animals for treatment of infections and prophylactic purposes and have also previously been used for growth enhancement (14). Antibiotics such as amoxicillins, penicillins, fluoroquinolones, sulfonamides and chlortetracycline are approved for use in cattle (14); most of these drugs or variants of these drugs are used in human medicine. The negative impacts of the widespread use of antibiotics in food animals prompted the World Health Organization to call for more prudent use of antibiotics (15). In an effort to control the spread of antibiotic resistance, the use of tetracycline as a growth promotant in animals was banned in Europe (16, 17) while the Center for Veterinary Medicines of Food and Drug Administration (FDA) has called for the elimination of antimicrobial drugs used for growth promotion by transitioning from over-the-counter-prescription (OTC) to veterinary feed directive status (VFD) (18). In 2016, 96% of all medically important antibiotics that were used in agricultural settings had

an OTC status whereas only 1% of medically important antibiotics were under VFD dispensing status (19).

The prevalence of antibiotic resistant STEC in cattle has been documented in several studies (20-22). For instance, Schroeder et al. detected antibiotic resistant STEC from cattle in different geographic locations and reported high frequencies of resistance to tetracycline and sulfamethoxazole, which are both widely administered to cattle (21). Other studies have examined the association between antibiotic use in food animals and antibiotic resistance rates in both humans and farm animals. In one study, identical antimicrobial resistance patterns were found in *E. coli* isolates from livestock and their farming families (23), highlighting the importance of elucidating the effects of agricultural antibiotic use on humans. In another study, Chantziaras et al. documented correlations between veterinary-associated antibiotic use in seven European countries and antibiotic resistance in commensal *E. coli* isolates from cattle, pigs and poultry (24).

While widespread use of antibiotics in the agricultural environment is common, the effects of antibiotic use are not well characterized, particularly for STEC given that the CDC has yet to characterize it as a serious antibiotic resistant threat (25). The use of antibiotics in food animals promotes selection of resistant foodborne pathogens that can have negative effects on human health. Such negative effects could be due to the consumption of food contaminated with resistant bacteria, direct contact with animals harboring resistant bacteria or mobile transfer of resistant genes to other clinically important pathogens. Consequently, numerous studies have called for the surveillance of antibiotic resistance in food animals worldwide (26, 27). Since cattle are an important reservoir for STEC, the emergence of antibiotic resistance in STEC in cattle and subsequent transmission to humans is very likely. Thus, this study was undertaken to determine

the frequency of antibiotic resistance in cattle-derived STEC isolates recovered from animals at six dairy farms and beef feedlots in Michigan.

MATERIALS AND METHODS

Sampling of herds and isolation of STEC

A subset of STEC isolates that were collected as part of the study carried out by Venegas Vargas et al. (11) were used in this study. Only a subset of isolates from the original study were examined here as some STEC isolates could not be recovered upon subculture. Although the *stx* profiles of the original isolates that were examined by Venegas-Vargas et al. were known, the original isolates either lost the bacteriophages encoding the *stx* genes or were not cultivable (28). Thus, these isolates were not included in this study. A total of 121 STEC isolates from 75 cattle from six herds in Michigan, collected between May 21st and August 27th of 2012, were tested as multiple isolates were recovered from the same animal. Overall, 50 STEC strains were isolated from dairy herds, while 71 STEC isolates were isolated from beef herds (Table 5.1)

DNA was extracted using the E.Z.N.A.® DNA/RNA Isolation Kit and isolates were confirmed for the presence of the *uidA* gene (1487 bp) encoding β-glucuronidase, *eae* (482 bp) encoding intimin and *stx1* (244 bp) and/or *stx2* (324bp) encoding Shiga toxin variants using multiplex PCR. The primers were used for each gene are provided in Table 5.2. The PCR cycle consisted of an initial denaturation step at 95°C for 10 min followed by 30 cycles of 15 s at 95°C, 15 s at 65°C and 30 s at 72°C, and a final extension for 3 min at 72°C. The serotypes of isolates were previously confirmed by molecular typing using PCR (28). On examining the STEC isolates recovered from 75 animals, ten animals had $\geq$1 distinct virulence gene/serotype profiles.

Antimicrobial susceptibility testing

The Kirby-Bauer disc diffusion test was used to determine antimicrobial susceptibility profiles of cattle isolates. The antibiotic susceptibility profiles to ampicillin (10 µg), trimethoprim-sulfamethoxazole/SXT (25 µg), ciprofloxacin (5 µg) and tetracycline (30 µg) were determined using Mueller Hinton agar plates. These antibiotics were selected because of their importance for human and veterinary medicine. After an incubation period of 18-20 hours at 37^{o}C, the zone of clearance around each Oxoid™ antibiotic disc was measured in millimeters. Strains were classified as resistant or susceptible as recommended by the Clinical Laboratory Standards Institute (CLSI) (29). *Escherichia coli* ATCC 25922 was used as the quality control strain for antimicrobial susceptibility testing in each experiment.

Data analysis

Isolate identification information and susceptibility profiles were managed using Microsoft Access and Excel. SAS version 9.3 (SAS Institute, Cary, NC, USA) and Epi Info™ 7 was used for all statistical analyses. The frequencies of antibiotic resistance were reported at both the animal level and at the isolate level. The frequency of antibiotic resistance at the animal level was defined as the number of cattle with at least one antibiotic resistant STEC divided by the total cattle in the study.

The cluster heat map was created using the seaborn.clustermap package and Python programming language. The cluster heat map was generated by hierarchical clustering of Euclidian distances using the Ward clustering method. If multiple STEC isolates with the same virulence gene profile and serotype were recovered from one animal, only one isolate was included when creating the cluster heat map. However, if isolates with different molecular profiles were isolated

from the same animal, both isolates were included in the cluster heat map. In this study, the molecular profile was considered to be different across isolates from the same animal if isolates differed based on the presence/absence of *eae* or if isolates had distinct *stx* gene profiles or by their serotypes.

RESULTS

Characteristics of cattle herds sampled for STEC

The six farms sampled for STEC isolates in this study were in six different counties in Michigan. A description of farm demographics, farm management and herd health management, which were compiled from questionnaires administered to farm owners and managers, is provided in Table 5.3. While farms 7D, 9D and 11B housed animals of the Holstein breed, animals in 10D and 12B were crossbred; 8B was the only farm that housed Angus cattle.

Of the six farms, farm 12B was the only farm reporting routine use of antibiotics (chlorotetracycline) in feed or water for new cattle upon their arrival. All farms, except 9D, used antibiotics such as ampicillin, ceftiofur (Excede®, Excenel®), florfenicol (Nuflor®) and macrolides gamithromycin (Zactran) & tulathromycin (Draxxin) for the treatment of respiratory diseases. For the treatment of foot infections, most farms administered antibiotics such as tetrayclines & oxytetracyclines (Oxytet 200), florfenicol (Nuflor®), ceftiofur (Excede®), tulathromycin (Draxxin); however, 10D was the only farm that used copper sulfate as the sole treatment for foot infections. Oxytetracycline (Oxytet 200) and tulathromycin (Draxxin) were used in 11B and 12B, respectively, for the treatment of arthritis. The herds with dairy cattle were reported to have been given antibiotics such as oxytetracyclines, lincosamide pirlimycin (Pirsue®), ceftiofur (SpectraMast® LC), cephapirin (ToDay®) and ampicillin (Polyflex®) for the treatment

of clinical mastitis and metritis. All six farms were reported to have contact with wildlife and birds including raccoons, rodents, skunks, deer, pigeons and starlings.

Virulence gene and serotype profiles of cattle-derived STEC isolates

Among the 121 *stx* positive isolates recovered from the subset of 75 animals included in the study, 32 (26.4%) were *stx1* positive, 59 (48.8%) were *stx2* positive and 30 (24.8%) carried both *stx1* and *stx2* genes. In addition, 53.7% (n=65) of all 121 STEC isolates also carried the *eae* gene. When stratified by type of production, dairy cattle had highest frequencies of STEC carrying only *stx1* (n=26, 52.0%) followed by *stx2* (n=16, 32.0%) and a combination of *stx1* and *stx2* (n=8, 16.0%). In beef herds, STEC carrying only *stx2* genes predominated (n=43, 60.6%) followed by isolates with both *stx1* and *stx2* genes (n=22, 30.9%) and *stx1* only isolates (n=6, 8.4%).The proportion of STEC isolates recovered from cattle also varied significantly by serotype (*p* value<0.001); most isolates were non-typeable (n=25, 30.5%) followed by O157 serotypes (n=22, 26.8%), O6 (n=16, 19.5%) and O98 (n=7, 8.5%). STEC isolates of multiple serotypes were observed when stratified by farm (Figure 5.1). Serotype O157 isolates were recovered from all farms except Farm 8B, although multiple serotypes such as O6, O103, O168 and O103 were recovered only from Farm 8B.

Isolate-level antimicrobial susceptibility profiles of STEC from cattle

Of the 121 STEC isolates recovered from cattle and tested for antimicrobial susceptibility in this study, 16.5% (n=20) were isolated from farm 7D, 28.1% (n=34) from 8B, 19.0% (n=23) from 9D, 5.8% (n=7) from 10D, 4.9% (n=6) from 11B and 25.6% (n=31) from farm 12B. Overall, 36 (29.7%) of all isolates were resistant to one or more antibiotics, with highest frequencies of resistance observed to tetracycline (n=36, 29.7%) and trimethoprim-

sulfamethoxazole (n=22, 18.2%); no resistance to ampicillin and ciprofloxacin were observed. Farm specific differences in antibiotic resistance were documented, with highest frequencies of antibiotic resistant STEC recovered from Farm 8B (n=27, 79.4%) followed by Farm 12B (n=8, 25.8%) and Farm 11B (n=1, 16.7%); isolates from farms 7D, 9D and 10D were pansusceptible (Figure 5.2).

When stratified by serotypes, non-O157 isolates (n=30, 50.0%) had significantly higher frequencies of antibiotic resistance than O157 isolates (n=0, 0.0%) (Fisher's exact *p* value<0.0001) (Table 5.4). Of the non-O157 serotypes, resistant isolates belonged to serotypes O6 (n=15, 93.7%), O168 (n=4, 100.0%) and non-typable (NT) (n=11, 44.0%). However, O6 isolates, which were recovered exclusively from Farm 8B, had significantly higher frequencies of resistance to one or more antibiotics (n=15, 93.7%) when compared to all other serotypes (n=15, 22.7%) (Chi-square *p* value<0.0001).

Significant differences were observed by *stx*-profile, with strains possessing *stx2* (n=33, 55.9%) more likely to be resistant than those possessing only *stx1* or a combination of *stx1* and *stx2* (n=3, 4.8%) (OR: 24.9; 95%CI: 7.0, 88.8). Of the non-O157 isolates, 28 (71.8%) of the resistant isolates carried the *stx2* gene, while both *stx1* and *stx2* positive isolates (n=2, 100.0%) were resistant; no *stx1* positive resistant non-O157 isolate were observed. Of the 65 strains that were *eae*-positive, none of them were resistant. When compared to *eae*-negative strains, *eae*-positive were less likely to be resistant (n = 0; 0.0%) than *eae*-negative strains (n = 36; 64.3%). Furthermore, when stratified by serotype, of all the 60 non-O157 isolates, all resistant non-O157 isolates were *eae*-negative (n=30, 78.95%).

Animal-level antimicrobial susceptibility profiles of STEC from cattle

Since multiple isolates, which may be duplicates, were isolated from the same animal, we also sought to determine the overall animal level frequency of antibiotic resistance. Overall, the animal-level frequency of antibiotic resistance in STEC, which was defined as resistance to at least one antibiotic, was 28% (n=21). Frequencies of animal-level resistance to tetracycline (n=21, 28.0%) were higher than those observed for trimethoprim-sulfamethoxazole (n=12, 16.0%) (Figure 5.3), although this difference was not significant (Chi square *p* value=0.076). No resistance to ampicillin and ciprofloxacin was observed. Only two antibiogram patterns were detected in this study (Figure 5.3). The most common pattern was resistance to tetracycline only (n=9, 12.0%) followed by resistance to both tetracycline and trimethoprim-sulfamethoxazole (n=12, 16.0%). Resistance to trimethoprim-sulfamethoxazole only were not observed.

When stratified by herd, the highest frequency of antibiotic resistance was observed in herds 8B (n=15, 75%) and 12B (n=5, 20.8%) followed by herd 11B (n=1, 16.6%), the three feedlots (Table 5.1). Herd 8B had significantly higher frequencies of resistance (Fisher's exact *p* value<0.001) when compared to herds 11B and 12B. In addition, animals belonging to Herd 8B were significantly more likely to be resistant to one or more antibiotic than isolates belonging to Herd 12B (OR: 11.4; 95% CI: 2.78, 46.80; Fisher's exact *p* value=0.0006). No antibiotic resistant STEC isolates were recovered from the three dairy herds examined in this study.

A cluster map shows clustering of antibiogram patterns by animals and herd (Figure 5.4). Two distinct clusters, Cluster A and Cluster B, were formed when animals were clustered according to the resistance levels of each STEC isolate. Cluster A, which comprised of animals with resistance to tetracycline and trimethoprim-sulfamethoxazole, only included animals from Herd 8B, Herd 11B and Herd 12B. A total of 53.8% of all STEC isolates with only tetracycline

resistance were recovered from animals in herds 8B and12B; one strain from 11B was resistant solely to tetracycline. Isolates with resistance to both drugs were isolated from animals belonging to Herd 8B only while none of the isolates were resistant to trimethoprim-sulfamethoxazole only. Compared with all other herds, isolates with any trimethoprim-sulfamethoxazole resistance were significantly more common in animals from Herd 8B (Fisher's exact *p value*<0.0001).

On examining the antibiotic resistant isolates in herds 8B, 11B and 12B, differences in serotypes of resistant STEC were observed (Figure 5.5 A). In Herd 8B, significantly higher proportions of resistant STEC isolates were O6, followed by O168 and non-typable (NT) strains (*p* value=0.0052). While all resistant STEC isolates recovered from cattle in 12B were non-typable, the serotype of the resistant isolate from Herd 11B was unknown. When stratified by serotype, all three farms had high proportions of antibiotic resistant isolates carrying the *stx2* gene (Figure 5.5 B). In Herd 8B, the proportions of resistant isolates with *stx*2 gene were significantly higher than those with *stx1* gene (*p* value<0.0001). Although not significant, the proportions of resistant STEC isolates in Herd 12B with *stx2* genes were higher (n=6. 75.0%) than those with a combination of *stx1* and *stx2* (n=2, 25.0%).

Variations in herd and farm management practices for herds were observed which may explain differences in antibiotic resistance frequencies (Table 5.5) Antibiotic resistant STEC isolates were more frequently recovered from beef operations (n=21, 42.0%) than dairy operations (n=0, 0.0%). Herds with cleaning practices reported significantly lower frequencies of antibiotic resistant isolates (n=6, 10.9%) compared to those not using any cleaning practices (n=15, 75.0%). Herd 8B, which had high frequencies of antibiotic resistant isolates, did not report the use of any cleaning practices unlike other herds. In addition, the use of more than one antibiotic for the treatment of infectious diseases such as foot infections, respiratory diseases and mastitis (only

applicable to dairy farms) significantly lowered the likelihood of antibiotic resistant STEC infections (OR:0.04; 95% CI: 0.01-0.15). Although tetracycline is administered in instances of foot infection in Herd 7D, no antibiotic resistant STEC isolates were recovered from this herd; although, this may be due to the small sample size of isolates tested. In Herd 11B, where one tetracycline resistant isolate was recovered, Oxytet 200 is administered for both foot infections and arthritis. Additionally, chlortetracycline is administered in feed and water in Herd 12B which may be a factor driving the high frequencies of tetracycline resistance (20.8%) observed in this herd. In Herd 8B, where STEC isolates with resistance to tetracycline and trimethoprim-sulfamethoxazole were recovered, none of these antibiotics are reported to have been administered. Since only six farms were sampled and the management practices varied considerably due to the type of cattle production system (beef or dairy), we were unable to conduct a multivariate analysis to identify factors associated with antibiotic resistant STEC infections in cattle.

DISCUSSION

Shiga toxin-producing *E. coli* is an important human pathogen resulting in 265,000 cases of food infections every year in the US (30). Animals such as sheep, cattle and pigs play an important role in the transmission of STEC infection to humans since they serve as reservoirs of STEC (1-3). In beef cattle, the prevalence rates of O157 and non-O157 STEC has been reported range between 0.2-27.8% and 2.1-70.1%, respectively (4), while worldwide prevalence rates for dairy cattle range from 0.2-48.8% for O157 and 0.4-74.0% for non-O157 isolates (5). In our prior Michigan study, the STEC prevalence rates varied by herd ranging from 10.9%-53.7% (11).

In the US, 5.7×10^6 kgs of medically important antibiotics and 4.2×10^6 kgs of non-medically important antibiotics are administered for production and therapeutic purposes in animals (19). The use of antibiotics in feed additives has generated a lot of controversy with advocates of their

use skeptical on whether these sub-inhibitory concentrations select for resistance (31). Indeed, Thomas et al. characterized the effect of feed additives on the resistome of feedlot cattle and did not observe any correlations between administration of feed antibiotics and the presence of antibiotic resistance genes (ARGs) in the gut microbiome (32). However, selection of resistant *E. coli* and *Salmonella enterica* have been shown to occur at very low antibiotic concentrations (33).

In cattle, tetracyclines such as chlortetracycline and oxytetracycline, are commonly administered. Indeed, in 2016, tetracyclines were the most widely used antibiotic in both food producing animals (cattle, swine etc.) and non-producing animals (horses, dogs etc.), with $5.8x10^6$ kgs (42%) of total antimicrobials sold in the US (19). In food animals, tetracyclines have been used for the treatment of respiratory infections, dermal and soft tissue infections, peritonitis, metritis and enteric infections (34). Tetracyclines have also been administered for growth promotion and prophylactic measures through drinking water or as feed additives (34, 35). Herd 12B, one of the three farms in our study with tetracycline resistant isolates, was the only farm that reported the use of chlortetracycline on arrival of new animals as a preventative measure; chlortetracycline was added to water at 2gms/head/day for five days every month. Additionally, Herd 11B, which had tetracycline resistance in 1 of the 6 strains examined, oxytetracycline (Oxytet 200) was given to cattle for the treatment of foot infections and arthritis. These results are consistent with a study conducted by Cha et al. showing high frequencies of tetracycline resistant *Campylobacter jejuni* isolates in cattle from herds 11B (Farm B) and 12B (Farm C) (36). Since, both STEC and *C. jejuni* were isolated from fecal samples of the same animals in these farms, these findings could suggest that the microbiome of livestock can serve as a potential reservoir of ARGs, resulting in horizontal transfer of ARGs between pathogenic and non-pathogenic bacteria. Indeed, tetracycline resistance genes, encoding efflux pumps and ribosomal protection proteins,

have been found on mobile genetic elements such as plasmids and conjugative transposons (37). By creating a selection pressure due to the constant administration of antibiotics, mutations in resistance genes may emerge or antibiotic resistance genes residing on mobile genetic elements may persist in the bacterial population (38).

It is noteworthy that one feedlot, Herd 8B, had significantly higher frequencies of resistance when compared to all other herds. Indeed, 8B was the only herd with isolates that were resistant to both trimethoprim-sulfamethoxazole and tetracycline. Interestingly, this herd only reported the use of florfenicol (Nuflor®) for treatment of respiratory diseases and foot infections; the current use of tetracyclines and sulfonamides were not reported. This finding suggests that resistance to tetracycline and trimethoprim-sulfamethoxazole is maintained in this herd in the absence of selective pressures created when using these two antibiotics. It is therefore possible that STEC isolates may contain genetic elements with resistance genes that confer a fitness advantage or are co-selected. The small plasmid p9123, which contains the *strA-strB-sul2-tet(A)* resistance gene cluster conferring resistance to streptomycin, sulfonamides and tetracycline (39), for instance, has been shown to confer a 4% fitness advantage to *E. coli* in the absence of selective pressures (40). Hence, a genomic analysis of the genetic determinants of antibiotic resistance and lineages of STEC circulating in the cattle reservoir, may provide additional information about the prevalence of ARGs and mobile genetic elements that persist in STEC.

Although we have previously documented ampicillin resistance in clinical STEC isolates (41) (Chapter 2), we did not detect resistance to ampicillin in any of the cattle-derived isolates examined, although farm 7D reported the use of ampicillin for the treatment of respiratory infections. This finding may indicate that ampicillin resistance observed in human isolates may exclusively be driven by the use of ampicillin and other β-lactam drugs in clinical settings (42,

43), or that our sampling scheme prevented detection of resistant isolates. Penicillin antibiotics are commonly used for the treatment of mastitis, respiratory diseases, diarrheal illnesses and other illnesses (44). Although ampicillin resistance has not been detected in any of the STEC isolates recovered from cattle in the current study, considering the use of penicillin antibiotics for therapeutic purposes in cattle and the ease of transmission of antibiotic resistance genes through populations, continuous monitoring of cattle isolates is warranted to track the emergence of ampicillin resistance in commensal and pathogenic bacteria. It is also possible that distinct antibiotic resistant STEC lineages may be circulating in clinical and community settings compared to those found in the farm environments. Further investigations into the genetic diversity of STEC isolates from cattle is warranted to further test this hypothesis.

Beef and dairy cattle differ in their operations with dairy calves raised intensively and administered more antibiotics than beef cattle (45). In dairy farms, respiratory and diarrheal infections are a problem in pre-weaned calves and therefore, young animals are given medicated milk replacers containing antibiotics such as oxytetracycline (46). In the US, 16% of all lactating dairy cows receive antibiotics for the treatment of clinical mastitis each year; however, most dairy cows receive prophylactic amounts of antibiotics such as penicillins and cephalosporins to prevent the development of future mastitis (47). According to the USDA, 15.8% antibiotics are used for the disease prevention and production purposes in the years 2007 and 2008 in beef cattle production; while in 2011, 73.4% of feedlots administered antibiotics in feed when the size of an operation exceeded 1000 animals (46). Bok et al. reported significantly higher antibiotic resistance rates to ampicillin, cefuroxime, ceftazidime, neomycin, tetracycline and sulfamethoxazole in commensal *E. coli* in dairy cattle (82.3%) than in beef cattle (58.5%) (48). Additionally, high prevalence of ARGs such as *bla-$_{TEM}$*, *tetA* and *bla-$_{CTX-M}$* were reported STEC O157 isolates from

dairy cattle in South Africa (49). However, in our present study, we observed high antibiotic resistance in isolates from beef cattle; no STEC isolate from dairy cattle were found to be resistant to any of the four antibiotics tested. However, considering how the gut of humans and animals is a large reservoir of antibiotic resistance genes (50, 51), the presence of antibiotic resistant isolates in other pathogenic and commensal bacterial species is very likely and merits further investigation. Considering the importance of the gut in transfer of resistance genes, numerous studies have looked at the resistome of animals as well as well as environmental sources in contact with animal production systems (52-54). Diet has also been shown to play an important role in influencing the ruminal resistome in beef cattle (55). Interestingly, studies have identified differences in rumen bacterial communities of cows belonging to different breeds (56, 57). For instance, Paz et al. identified distinct clustering of bacterial communities by breed suggesting that Holstein and Jersey cows have different bacterial communities in the rumen (56). In our study, highest frequencies of resistant STEC isolates were recovered from Angus cattle in Herd 8B; thus, resistome differences by breed could serve as a possible explanation for this finding. Moreover, while our study is the first to determine antibiotic resistance in STEC isolates from cattle, a greater sample size of isolates from cattle, over longer periods of time, may be required to be tested to discern differences between STEC resistance in dairy and beef cattle operations in Michigan. Additionally, specific herd and farm management practices in the beef feedlots could also be responsible for high frequencies of resistance observed. For instance, the feedlot housing conditions in Herd 11B and 12B, which result in close contact between animals, may aid in the transmission of antibiotic resistant STEC between animals. While studies have identified risk factors associated with antibiotic resistant bacteria in cattle (58, 59), factors have yet to be identified for antibiotic resistant STEC. Duse et al., for instance, noted that feeding preweaned dairy calves with milk from cows treated with

antimicrobials during lactation was a risk factor of antibiotic resistance in *E. coli* in preweaned dairy calves in Sweden (58), while Berge et al. identified higher resistance levels in *E. coli* isolates from cattle in conventional versus organic farms (59). Age has also been identified as a factor influencing antibiotic resistance frequencies, with younger animals documented to have higher frequencies of antibiotic resistant organisms than older animals (60) and has been attributed to differences in exposure to antibiotics for treatment and growth promotion. In our study, although we did not sample calves, all the beef cattle were close to a year in age, compared to dairy cattle which were older than one year.

Although this study is the first to report antibiotic resistance frequencies in cattle-derived STEC isolates from Michigan, there are a few limitations. First, since this study was designed to be a cross-sectional study with sampling based on convenience, we acknowledge that the prevalence of STEC in cattle and thus, resistance frequencies may not represent the true frequencies of antibiotic resistant STEC in cattle in Michigan. Furthermore, while we did observe high frequencies of antibiotic resistance in herds 8B and 12B, we are unable to identify factors associated with resistant infections in these herds specifically due to the small sample sizes. Further investigations into herd and farm management practices in these herds, may provide valuable information about practices that can help lower the prevalence of antibiotic resistant isolates in cattle.

Considering the importance of cattle as a reservoir of STEC infections and the emergence of antibiotic resistance in both O157 and non-O157 serotypes of STEC, continuous monitoring of the agricultural environment for resistance and lineages associated with antibiotic resistance may help in the design of novel intervention strategies and policies to control the spread of antibiotic resistance.

APPENDIX

Table 5.1. Number of STEC isolates recovered from each farm and frequencies of antibiotic resistance observed in Shiga toxin-producing *E. coli* (STEC) in six cattle herds in Michigan

Herd	Type of production system	Collection date	Total animals	No. STEC tested for resistance profiles	No. of cattle STEC isolates were recovered from	% animals with resistant STEC
7D	Dairy	5/29/2012	12000	20	10	0.0%
8B	Beef	6/19/2012	54	34	20	75.0%
9D	Dairy	7/9/2012	243	23	10	0.0%
10D	Dairy	7/23/2012	530	7	5	0.0%
11B	Beef	8/13/2012	83	6	6	16.6%
12B	Beef	8/27/2012	75	31	24	20.8%

Table 5.2. Primers used in the study for the detection of *uidA*, *stx* and *eae* genes in isolates from cattle

Gene	Primer	Sequence	Size (bp)
uidA	*uidA*_FP	5’ ATGCCAGTCCAGCGTTTTTGC 3’	
	*uidA_R*P	5’ AAAGTGTGGGTCAATAATCAGGAAGTG 3’	1487 bp
stx1	*stx1*_FP	5’ CGATGTTACGGTTTGTTACTGTGACAGC 3’	
	*stx1*_RP	5’ AATGCCACGCTTCCCAGAATTG 3’	244 bp
stx2	*stx2*_FP	5’ GTTTTGACCATCTTCGTCTGATTATTGAG 3’	
	*stx2*_RP	5’ AGCGTAAGGCTTCTGCTGTGAC 3’	324 bp
eae	*eae*_FP	5’ TCAATGCAGTTCCGTTATCAGTT 3’	
	*eae*_RP	5’ GTAAAGTCCGTTACCCCAACCTG 3’	482 bp

Table 5.3. Questionnaire derived information about the six farms in Michigan selected for this study

		7D	8B	9D	10D	11B	12B
Demographic	**Operation type**	Dairy	Beef	Dairy	Dairy	Beef	Beef
	Breed	Holstein	Angus	Holstein	Crossbred	Holstein	Crossbred
Preventative measures	**Antibiotic use in feed or water**	No	No	No	No	No	Yes (Chlortetracycline)
	Any direct fed microbials	No	No	No	No	No	Yes (Yeast mineral package)
	Antiparasitic	No (but Panacur®, Cydectin® on occasion)	No	No (Cydectin® in the future)	Yes (Cydectin®)	Yes (Dectomax®)	Yes (Dectomax®)
	Rumensin in the feed	Yes	No	No	No	Yes	Yes
Treatment	**Respiratory Disease**	Ceftiofur, Ampicilin	Nuflor®	No	Excede®, Excenel®, Nuflor® (Calf)	Excede®, Nuflor®, Zactran, Draxxin	Draxxin
	Foot infection	Tetracycline (Topic)	Nuflor®	Copper sulfate= footbath, Oxytetracycline = HCI powder, Excede®	Copper sulfate	Oxytet 200	Draxxin
	Arthritis	Unknown	No	Unknown	Unknown	Oxytet 200	Draxxin
	Clinical mastitis/metritis	Pirsue®, SpectraMast®	N/A	SpectraMast® LC, ToDay®	ToDay®, Oxytetracycline, Polyflex®	N/A	N/A

Table 5.3 (cont'd)

Contact with other species	**Fly control**	Premise spray	Ear tags; None; Pour-on insecticide	Pour-on insecticide; Premise spray	Yes (Premise spray)	No	No
	Dogs	No	Yes	No	Yes	No	Yes
	Cats	Yes	Yes	Yes	No	Yes	Yes
	Birds	Yes	Yes	Yes	Yes (Starlings, Pigeons)	Yes (Sparrows, Starlings)	Yes (Sparrows, Starlings, pigeons)
	Other animals	Yes (raccoons, rodents, skunks, opossum)	Yes (raccoons, rodents, deer, skunks, opossum)	Yes (raccoons, rodents, deer, skunks, opossum)	Yes (raccoons, rodents, deer)	Yes (raccoons, rodents, skunks)	Yes (raccoons, rodents, skunks, opossum, weasel)
Cleaning	**Method**	Scrape; Spread lime; Vacuum tank	None	Scrape; Wash/Power Wash	Scrape; Wash/Power wash, spread lime	Wash/Power Wash	Spray a disinfectant
	Feedbunks	Once a day		winter twice a week, summer once a month	Once a week	When needed	
	Waterers	Once a week	Once per day	Once a week	Once a week	20 per month	
Environment	**Temperature**	71°F (61-80°F)	78°F (66-90°F)	76°F (63-89°F)	85°F (73-97°F)	68°F (62-73°F)	75°F (65-84°F)

Table 5.4. Antibiotic resistance in 121 Shiga toxin-producing *E. coli* (STEC) isolates recovered from cattle, by serotype. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline

Serotype	Total Isolates*	Any Resistance	AMP Resistance	CIP Resistance	SXT Resistance	TET Resistance
		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
O157	22	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Non-O157	60	30 (50.0%)	0 (0.0%)	0 (0.0%)	19 (41.0%)	30 (50.0%)
NT	25	11 (44.0%)	0 (0.0%)	0 (0.0%)	4 (16.0%)	11 (44.0%)
O103	2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
O121	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
O168	4	4 (100.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	4 (100.0%)
O169	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
O26	1	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
O45	3	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
O6	16	15 (93.7%)	0 (0.0%)	0 (0.0%)	15 (93.7%)	15 (93.7%)
O98	7	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

Isolate numbers for individual antibiotics do not always add up to the total number of isolates with any resistance because some isolates were resistant to more than one antibiotic.

‡ 39 isolates had unknown serotypes and were excluded from this analysis.

Table 5.5. Univariate analysis of factors associated with antibiotic resistant Shiga toxin-producing *E. coli* (STEC) in cattle belonging to six herds in Michigan

Characteristic	Total Strains	No. (%) Resistant	OR (95% CI) †	*p* value‡
Operation Type				
Beef	50	21 (42.0%)	-	<0.0001
Dairy	25	0 (0.0%)		-
Breed				
Holstein	26	1 (3.8%)	1.0	-
Angus	20	15 (75.0%)	75.0 (7.9-704.8)	<0.0001
Crossbred	29	5 (17.2%)	5.2 (0.57-47.9)	0.10
Antibiotic use in feed or water				
Yes	24	5 (20.8%)	0.6 (0.18-1.82)	0.42
No	51	16 (31.2%)	1.0	-
Infectious Disease Treatment				
One antibiotic	20	15 (75.0%)	1.0	-
Multiple	55	6 (10.9%)	0.04 (0.01-0.15)	<0.0001
Cleaning				
Yes	55	6 (10.9%)	0.04 (0.01-0.15)	<0.0001
No	20	15 (75.0%)	1.0	-

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells

Figure 5.1. Distribution of serotypes of Shiga toxin-producing *E. coli* (STEC) recovered from cattle farms in Michigan

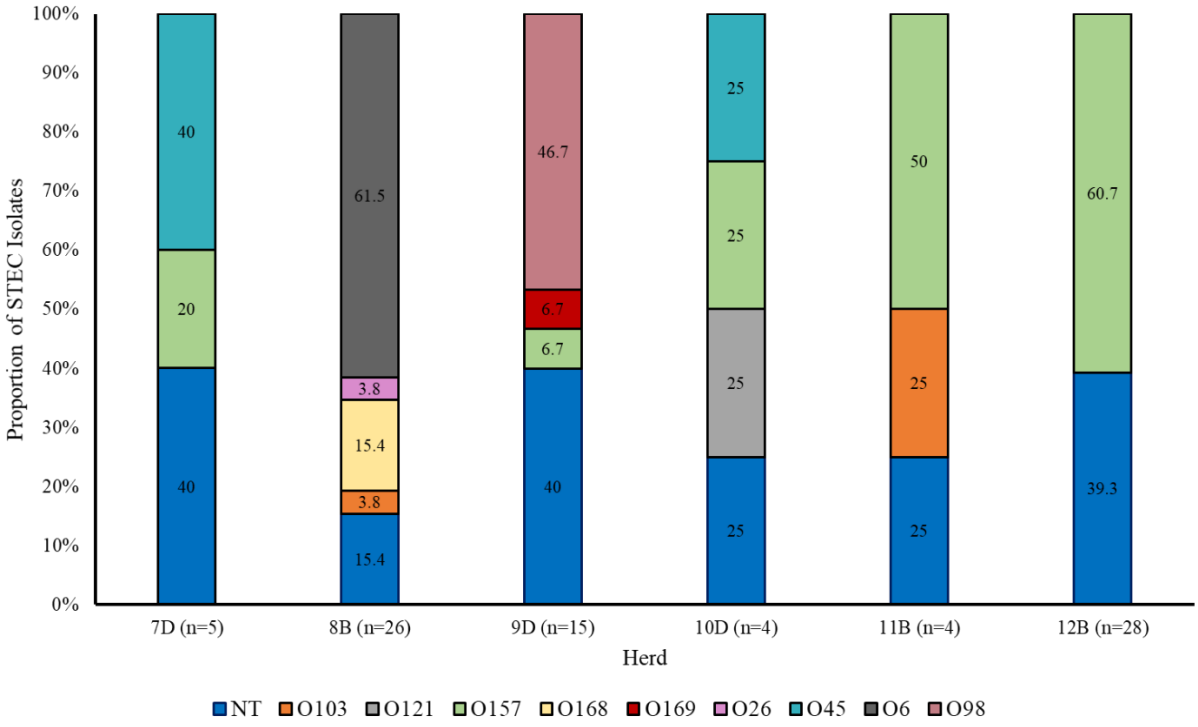


Figure 5.2. Frequency antimicrobial resistance in Shiga toxin-producing *E. coli* (STEC) isolates recovered from cattle (n=121), stratified by farm. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline

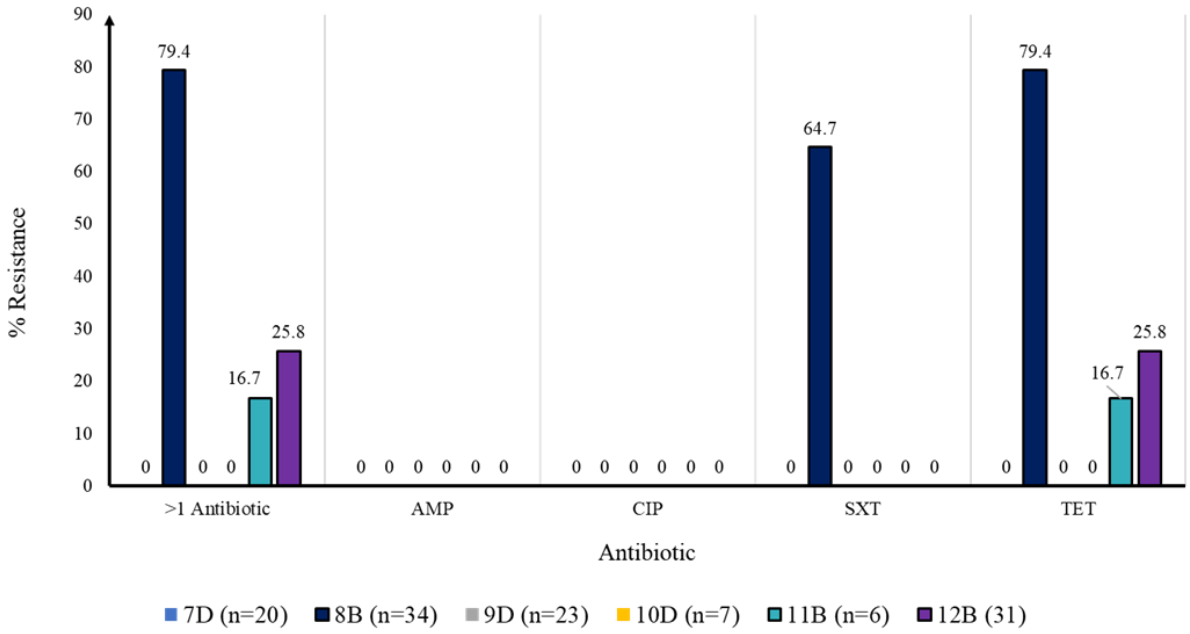


Figure 5.3. Proportion of resistant and susceptible Shiga toxin-producing *E. coli* (STEC) isolate containing animals (n=75) in Michigan. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline

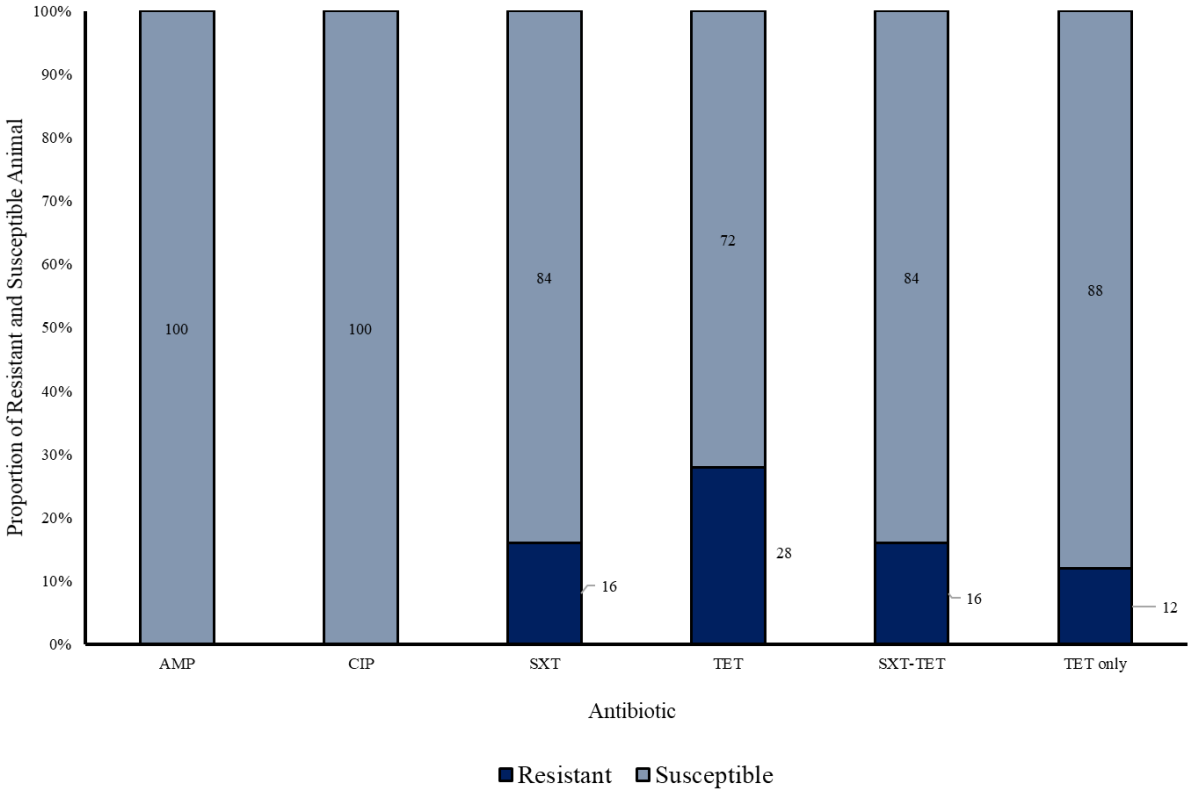


Figure 5.4. Cluster analysis of antibiotic susceptibility patterns in Shiga toxin-producing *E. coli* (STEC) isolates (n=85) from cattle (n=75) in Michigan. The bottom matrix border shows the different types of antibiotics that were tested in the study. The color indicates the susceptibility gradient with dark blue indicating lower zone of clearance (resistant) and light blue indicating higher zone of clearance (susceptible). The dendrogram on the left margin indicates the relationship between animals in terms of the susceptibility testing profiles of STEC isolates. Abbreviation: AMP, ampicillin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline

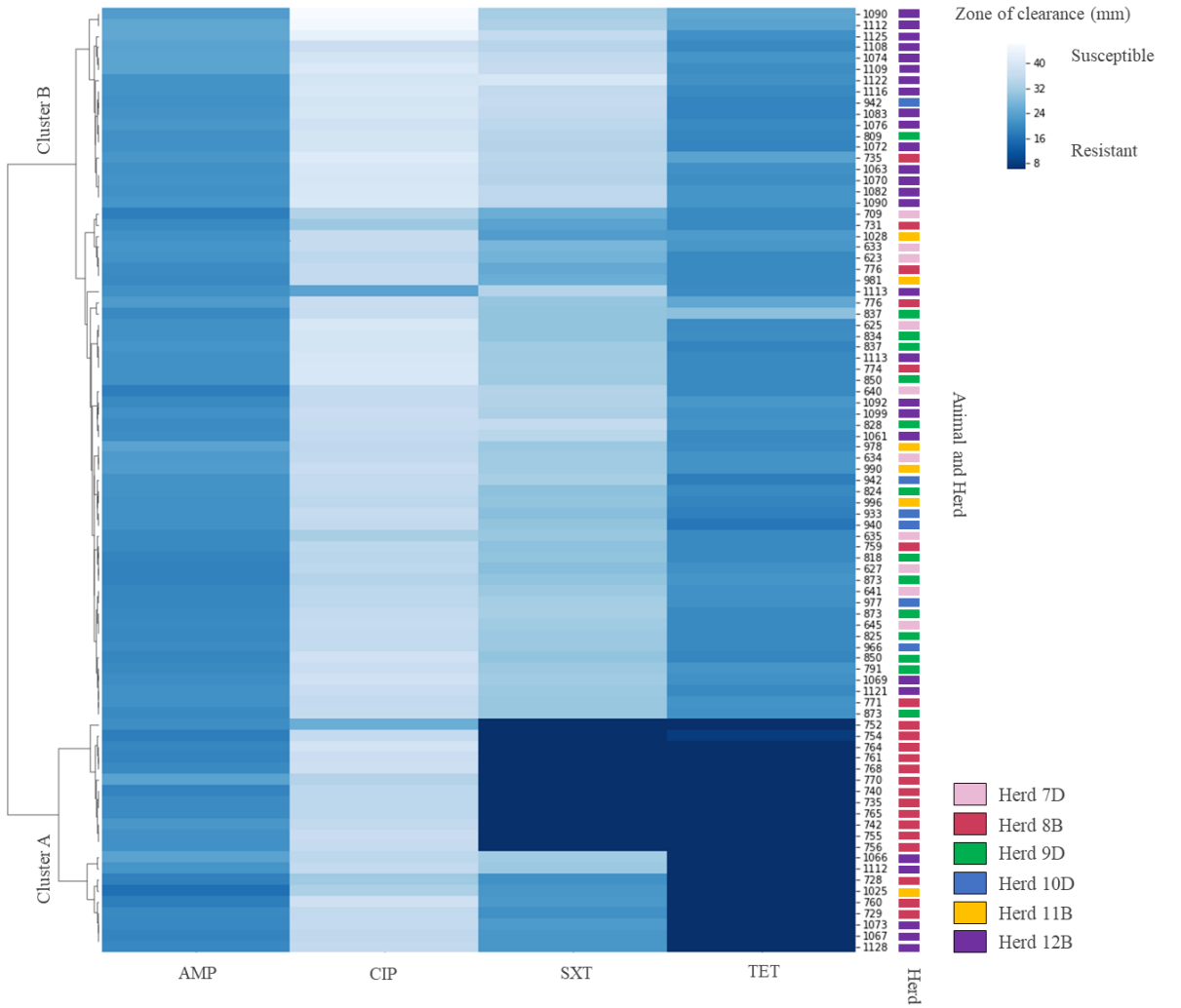
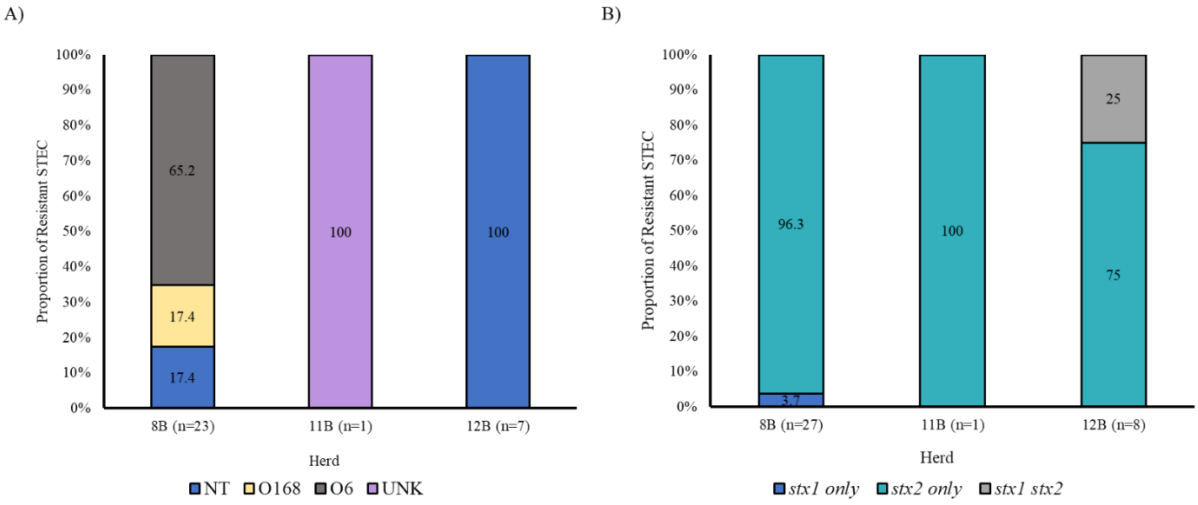


Figure 5.5. Proportion of resistant Shiga toxin-producing *E. coli* (STEC) recovered from herds 8B, 11B and 12B. A) Distribution of resistant isolates by serotype B) Distribution of resistant isolates by *stx* status



REFERENCES

REFERENCES

1. Zschock M, Hamann HP, Kloppert B, Wolter W. Shiga-toxin-producing *Escherichia coli* in faeces of healthy dairy cows, sheep and goats: prevalence and virulence properties. Lett Appl Microbiol. 2000;31(3):203-8.

2. Beutin L, Geier D, Steinruck H, Zimmermann S, Scheutz F. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J Clin Microbiol. 1993;31(9):2483-8.

3. Kobayashi H, Shimada J, Nakazawa M, Morozumi T, Pohjanvirta T, Pelkonen S, Yamamoto K. Prevalence and characteristics of shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl Environ Microbiol. 2001;67(1):484-9. doi: 10.1128/AEM.67.1.484-489.2001.

4. Hussein HS, Bollinger LM. Prevalence of Shiga toxin-producing *Escherichia coli* in beef cattle. J Food Prot. 2005;68(10):2224-41.

5. Hussein HS, Sakuma T. Prevalence of shiga toxin-producing Escherichia coli in dairy cattle and their products. J Dairy Sci. 2005;88(2):450-65.

6. Cobbold RN, Rice DH, Szymanski M, Call DR, Hancock DD. Comparison of shiga-toxigenic *Escherichia coli* prevalences among dairy, feedlot, and cow-calf herds in Washington State. Appl Environ Microbiol. 2004;70(7):4375-8. doi: 10.1128/AEM.70.7.4375-4378.2004.

7. Centers for Disease Control and Prevention. Multistate Outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 Infections Linked to Ground Beef (Final Update). 2014.

8. Schlager S, Lepuschitz S, Ruppitsch W, Ableitner O, Pietzka A, Neubauer S, Stöger A, Lassnig H, Mikula C, Springer B, Allerberger F. Petting zoos as sources of Shiga toxin-producing *Escherichia coli* (STEC) infections. Int J Med Microbiol. 2018. doi: https://doi.org/10.1016/j.ijmm.2018.06.008.

9. Rivas M, Sosa-Estani S, Rangel J, Caletti MG, Valles P, Roldan CD, Balbi L, Marsano de Mollar MC, Amoedo D, Miliwebsky E, Chinen I, Hoekstra RM, Mead P, Griffin PM. Risk factors for sporadic Shiga toxin-producing *Escherichia coli* infections in children, Argentina. Emerg Infect Dis. 2008;14(5):763-71. doi: 10.3201/eid1405.071050.

10. McPherson M, Lalor K, Combs B, Raupach J, Stafford R, Kirk MD. Serogroup-specific risk factors for Shiga toxin-producing *Escherichia coli* infection in Australia. Clin Infect Dis. 2009;49(2):249-56. doi: 10.1086/599370.

11. Venegas-Vargas C, Henderson S, Khare A, Mosci RE, Lehnert JD, Singh P, Ouellette LM, Norby B, Funk JA, Rust S, Bartlett PC, Grooms D, Manning SD. Factors Associated with Shiga Toxin-Producing *Escherichia coli* Shedding by Dairy and Beef Cattle. Appl Environ Microbiol. 2016;82(16):5049-56. doi: 10.1128/AEM.00829-16.

12. Cho S, Fossler CP, Diez-Gonzalez F, Wells SJ, Hedberg CW, Kaneene JB, Ruegg PL, Warnick LD, Bender JB. Cattle-level risk factors associated with fecal shedding of Shiga toxin-encoding bacteria on dairy farms, Minnesota, USA. Can J Vet Res. 2009;73(2):151-6.

13. Chase-Topping ME, McKendrick IJ, Pearce MC, MacDonald P, Matthews L, Halliday J, Allison L, Fenlon D, Low JC, Gunn G, Woolhouse MEJ. Risk Factors for the Presence of High-Level Shedders of *Escherichia coli* O157 on Scottish Farms. J Clin Microbiol. 2007;45(5):1594.

14. McEwen SA, Fedorka-Cray PJ. Antimicrobial use and resistance in animals. Clin Infect Dis. 2002;34 s93-s106.

15. World Health Organization. Division of Emerging and other Communicable Diseases Surveillance and Control. The Medical impact of the use of antimicrobials in food animals: report of a WHO meeting, Berlin, Germany, 13-17 October 1997.

16. Castanon JIR. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. Poultry Science. 2007;86(11):2466-71. doi: 10.3382/ps.2007-00249.

17. Marshall BM, Levy SB. Food Animals and Antimicrobials: Impacts on Human Health. Clin Microbiol Rev. 2011;24(4):718.

18. U.S. Food and Drug Administration. Guidance for Industry: New Animal Drugs and New Animal Drug Combination Products Administered in or on Medicated Feed or Drinking Water of Food-Producing Animals: Recommendations for Drug Sponsors for Voluntarily Aligning Product Use Conditions with GFI #2092017.

19. U.S. Food and Drug Administration Center for Veterinary Medicine. Summary Report On Antimicrobials Sold or Distributed for Use in Food-Producing Animals. 2017.

20. Meng J, Zhao S, Doyle MP, Joseph SW. Antibiotic resistance of *Escherichia coli* O157:H7 and O157:NM isolated from animals, food, and humans. J Food Prot. 1998;61(11):1511-4.

21. Schroeder CM, Zhao C, DebRoy C, Torcolini J, Zhao S, White DG, Wagner DD, McDermott PF, Walker RD, Meng J. Antimicrobial resistance of *Escherichia coli* O157 isolated from humans, cattle, swine, and food. Appl Environ Microbiol. 2002;68(2):576-81.

22. Schroeder CM, Meng J, Zhao S, DebRoy C, Torcolini J, Zhao C, McDermott PF, Wagner DD, Walker RD, White DG. Antimicrobial resistance of *Escherichia coli* O26, O103, O111, O128, and O145 from animals and humans. Emerg Infect Dis. 2002;8(12):1409-14. doi: 10.3201/eid0812.020070.

23. Fein D, Burton G, Tsutakawa R, Blenden D. Matching of antibiotic resistance patterns of *Escherichia coli* of farm families and their animals. J Infect Dis. 1974;130:274-9.

24. Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother. 2014;69(3):827-34. doi: 10.1093/jac/dkt443.

25. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

26. Thanner S, Drissner D, Walsh F. Antimicrobial Resistance in Agriculture. mBio. 2016;7(2).

27. Aarestrup FM. Monitoring of antimicrobial resistance among food animals: principles and limitations. J Vet Med B Infect Dis Vet Public Health. 2004;51(8-9):380-8. doi: 10.1111/j.1439-0450.2004.00775.x.

28. Singh P, Sha Q, Lacher DW, Del Valle J, Mosci RE, Moore JA, Scribner KT, Manning SD. Characterization of enteropathogenic and Shiga toxin-producing *Escherichia coli* in cattle and deer in a shared agroecosystem. Front Cell Infect Microbiol. 2015;5:29. doi: 10.3389/fcimb.2015.00029.

29. Clinical & Laboratory Standards Insitute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

30. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1.

31. Chattopadhyay MK. Use of antibiotics as feed additives: a burning question. Front Microbiol. 2014;5:334. doi: 10.3389/fmicb.2014.00334.

32. Thomas M, Webb M, Ghimire S, Blair A, Olson K, Fenske GJ, Fonder AT, Christopher-Hennings J, Brake D, Scaria J. Metagenomic characterization of the effect of feed additives on the gut microbiome and antibiotic resistome of feedlot cattle. Sci Rep. 2017;7(1):12257. doi: 10.1038/s41598-017-12481-6.

33. Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, Andersson DI. Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. PLOS Pathogens. 2011;7(7):e1002158. doi: 10.1371/journal.ppat.1002158.

34. Granados-Chinchilla F, Rodríguez C. Tetracyclines in Food and Feedingstuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications. J Anal Methods Chem. 2017;2017:1315497. doi: 10.1155/2017/1315497.

35. United States Department of Agriculture Animal and Plant Health Inspection Service. Antibiotic Use on U.S. Dairy Operations, 2002 and 2007. 2008.

36. Cha W, Mosci RE, Wengert SL, Venegas Vargas C, Rust SR, Bartlett PC, Grooms DL, Manning SD. Comparing the Genetic Diversity and Antimicrobial Resistance Profiles of *Campylobacter jejuni* Recovered from Cattle and Humans. Front Microbiol. 2017;8:818. doi: 10.3389/fmicb.2017.00818.

37. Speer BS, Shoemaker NB, Salyers AA. Bacterial resistance to tetracycline: mechanisms, transfer, and clinical significance. Clin Microbiol Rev. 1992;5(4):387-99.

38. Andersson DI, Hughes D. Persistence of antibiotic resistance in bacterial populations. FEMS Microbiol Rev. 2011;35(5):901-11. doi: 10.1111/j.1574-6976.2011.00289.x.

39. Anantham S, Hall RM. pCERC1, a small, globally disseminated plasmid carrying the *dfrA14* cassette in the *strA* gene of the *sul2-strA-strB* gene cluster. Microb Drug Resist. 2012;18(4):364-71. doi: 10.1089/mdr.2012.0008.

40. Enne VI, Bennett PM, Livermore DM, Hall LM. Enhancement of host fitness by the *sul2*-coding plasmid p9123 in the absence of selective pressure. J Antimicrob Chemother. 2004;53(6):958-63. doi: 10.1093/jac/dkh217.

41. Mukherjee S, Mosci RE, Anderson CM, Snyder BA, Collins J, Rudrik JT, Manning SD. Antimicrobial Drug-Resistant Shiga Toxin-Producing *Escherichia coli* Infections, Michigan, USA. Emerg Infect Dis. 2017;23(9):1609-11. doi: 10.3201/eid2309.170523.

42. Kofke-Egger H, Udow-Phillips, Marianne. Antibiotic Prescribing and Use. Center for Healthcare Research and Transformation. 2011.

43. Nelson JM, Griffin PM, Jones TF, Smith KE, Scallan E. Antimicrobial and antimotility agent use in persons with shiga toxin-producing *Escherichia coli* O157 infection in foodnet sites. Clin Infect Dis. 2011;52(9):1130-2.

44. De Briyne N, Atkinson J, Borriello SP, Pokludová L. Antibiotics used most commonly to treat animals in Europe. Vet Rec. 2014;175(13):325.

45. Call DR, Davis MA, Sawant AA. Antimicrobial resistance in beef and dairy cattle production. Anim Health Res Rev. 2008;9(2):159-67. doi: 10.1017/s1466252308001515.

46. United States Department of Agriculture Economic Research Service. Economics of Antibiotic Use in U.S. Livestock Production. 2015.

47. Landers TF, Cohen B, Wittum TE, Larson EL. A Review of Antibiotic Use in Food Animals: Perspective, Policy, and Potential. Public Health Rep. 2012;127(1):4-22.

48. Bok E, Mazurek J, Stosik M, Wojciech M, Baldy-Chudzik K. Prevalence of Virulence Determinants and Antimicrobial Resistance among Commensal *Escherichia coli* Derived from Dairy and Beef Cattle. Int J Environ Res Public Health. 2015;12(1):970-85. doi: 10.3390/ijerph120100970.

49. Iweriebor BC, Iwu CJ, Obi LC, Nwodo UU, Okoh AI. Multiple antibiotic resistances among Shiga toxin producing *Escherichia coli* O157 in feces of dairy cattle farms in Eastern Cape of South Africa. BMC Microbiol. 2015;15:213. doi: 10.1186/s12866-015-0553-y.

50. Lerner A, Matthias T, Aminov R. Potential Effects of Horizontal Gene Exchange in the Human Gut. Front Immunol. 2017;8:1630-. doi: 10.3389/fimmu.2017.01630.

51. van Schaik W. The human gut resistome. Philos Trans R Soc Lond B Biol Sci. 2015;370(1670):20140087-. doi: 10.1098/rstb.2014.0087.

52. Wichmann F, Udikovic-Kolic N, Andrew S, Handelsman J. Diverse Antibiotic Resistance Genes in Dairy Cow Manure. mBio. 2014;5(2).

53. Noyes NR, Yang X, Linke LM, Magnuson RJ, Cook SR, Zaheer R, Yang H, Woerner DR, Geornaras I, McArt JA, Gow SP, Ruiz J, Jones KL, Boucher CA, McAllister TA, Belk KE, Morley PS. Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. Sci Rep. 2016;6:24645-. doi: 10.1038/srep24645.

54. Noyes NR, Yang X, Linke LM, Magnuson RJ, Dettenwanger A, Cook S, Geornaras I, Woerner DE, Gow SP, McAllister TA, Yang H, Ruiz J, Jones KL, Boucher CA, Morley PS, Belk KE. Resistome diversity in cattle and the environment decreases during beef production. eLife. 2016;5:e13195-e. doi: 10.7554/eLife.13195.

55. Auffret MD, Dewhurst RJ, Duthie C-A, Rooke JA, John Wallace R, Freeman TC, Stewart R, Watson M, Roehe R. The rumen microbiome as a reservoir of antimicrobial resistance and pathogenicity genes is directly affected by diet in beef cattle. Microbiome. 2017;5(1):159-. doi: 10.1186/s40168-017-0378-z.

56. Paz HA, Anderson CL, Muller MJ, Kononoff PJ, Fernando SC. Rumen Bacterial Community Composition in Holstein and Jersey Cows Is Different under Same Dietary Condition and Is Not Affected by Sampling Method. Front Microbiol. 2016;7:1206-. doi: 10.3389/fmicb.2016.01206.

57. Austin KJ, Cunningham HC, Powell SR, Carpenter KT, Cammack KM. Investigation of maternal breed and rearing type on the calf rumen microbiome from day 28 through

weaning. Translational Animal Science. 2018;2(suppl_1):S125-S9. doi: 10.1093/tas/txy034.

58. Duse A, Waller KP, Emanuelson U, Unnerstad HE, Persson Y, Bengtsson B. Risk factors for antimicrobial resistance in fecal *Escherichia coli* from preweaned dairy calves. J Dairy Sci. 2015;98(1):500-16. doi: 10.3168/jds.2014-8432.

59. Berge AC, Hancock DD, Sischo WM, Besser TE. Geographic, farm, and animal factors associated with multiple antimicrobial resistance in fecal *Escherichia coli* isolates from cattle in the western United States. J Am Vet Med Assoc. 2010;236(12):1338-44. doi: 10.2460/javma.236.12.1338.

60. Khachatryan AR, Hancock DD, Besser TE, Call DR. Role of calf-adapted *Escherichia coli* in maintenance of antimicrobial drug resistance in dairy calves. Appl Environ Microbiol. 2004;70(2):752-7. doi: 10.1128/AEM.70.2.752-757.2004.

CHAPTER 6

Frequency and Epidemiologic Factors Associated with Clinical Antibiotic Resistant Non-Typhoidal *Salmonella* (NTS) Infections in Michigan, 2011-2014

ABSTRACT

Non-typhoidal *Salmonella* (NTS) are important enteric pathogens causing over 1 million food-illnesses in the US annually. The widespread emergence of antibiotic resistance in NTS isolates has limited the availability of antibiotics that can be used for therapy. Since Michigan is not part of the FoodNet surveillance system, few studies have quantified antibiotic resistance frequencies and identified risk factors for NTS infections. We obtained 198 clinical NTS isolates via active surveillance at four Michigan hospitals from 2011 to 2014 for classification of serovars and susceptibility to 24 antibiotics using broth microdilution. To identify risk factors for NTS infections and resistant NTS infections, we used case information and epidemiological data from the Michigan Disease Surveillance System (MDSS). In all, 30 (15.2%) isolates were resistant to ≥1 antibiotic and 15 (7.5%) were resistant to ≥3 antimicrobial classes. An increasing trend in the frequency of tetracycline and multidrug resistance was observed over the four-year period, and resistant infections were significantly associated with longer hospital stays. The mean hospital stay was 5.9 days for patients with resistant isolates relative to 4 days for those with susceptible isolates. Multinominal logistic regression identified drinking bottled water at home to be independently associated with Enteritidis (Odds Ratio (OR):6.1; 95% Confidence Interval (CI): 1.25-30.18) and Typhimurium (OR: 6.9; 95% CI: 1.23-39.29) infections, while infection with serovars other than Enteritidis (OR: 4.3, 95% CI: 1.18-15.62) and fall, winter and spring seasons (OR: 3.5; 95% CI1.23-9.72) were independently associated with resistance. Together, these findings demonstrate the importance of surveillance, monitoring resistance frequencies, and identifying risk factors that help in the development of new prevention strategies.

INTRODUCTION

The Gram-negative pathogen, *Salmonella enterica*, is an important public health concern, resulting in about 93.8 million cases of food infections globally (1). In 2015, the non-typhoidal *S. enterica* (NTS) serovars were reported to be one of the leading causes of deaths due to diarrhea, with 90,300 deaths reported (2). NTS infections were also estimated to result in 70 disability-adjusted life years (DALY) lost/100,000 persons worldwide in 2010 (3). In the U.S., NTS causes 1.2 million infections per year with 23,000 hospitalizations and 450 deaths (4). Furthermore, *Salmonella* infections have the highest mean cost of illness among all foodborne infections (5), and geographical differences in serovar prevalence have been documented (6). In Europe and Asia, for instance, *S.* Enteritidis was the leading cause of clinical infections in the year 2002, whereas *S.* Typhimurium was the highest in North America followed by *S.* Enteritidis, *S.* Newport and *S.* Heidelberg. Indeed, the Typhimurium and Enteritidis serovars are the leading causes of enterocolitis and, in severe cases, bacteremia (7). Infections with NTS can cause nausea, vomiting, abdominal pain, myalgias (muscle pain) and arthralgias (joint pain), while hepatomegaly (liver enlargements) and splenomegaly (spleen enlargements) can develop in a subset of cases (7) and systemic infections can occur in immunocompromised patients (8).

NTS has been frequently isolated from commercially raised chickens and other poultry (9, 10) and contact with cattle, pigs, horses and other domestic animals are important risk factors for NTS infections (9). In addition, approximately 74,000 infections of *Salmonella* infections in the US were attributed to reptile and amphibian exposures (11); contact with reptiles and cats was associated with salmonellosis in a prior Michigan study (12). Other studies, however, have identified risk factors for infection with specific serovars. One study in the Netherlands, for example, found consumption of raw eggs and products containing raw eggs to be linked to

Salmonella Enteritidis infections, while exposure to raw meat and playing in a sandbox were risk factors for *S.* Typhimurium infections (13). Prior history of antibiotic use, living on a livestock farm, and international travel were also identified as risk factors for *S.* Typhimurium infections in Canada (14). Indeed, NTS isolates have been recovered from environmental sources including water and soil and can often survive in these environments for extended periods of time (15-19). Taken together, these studies indicate the importance of the environment as a source of *Salmonella* infections in humans.

Drug-resistant NTS infections have also emerged and are increasing in frequency in the U.S. resulting in high hospitalization rates and approximately $365,000,000 in medical costs (20). The fluoroquinolones, third generation cephalosporins, penicillins, macrolides and trimethoprim-sulfamethoxazole are commonly prescribed for the treatment of salmonellosis, particularly in patients with immunocompromising conditions, young children and the elderly (21). Importantly, drug resistant *Salmonella* infections have been linked to more severe disease outcomes, including bloodstream infections as well as hospitalization (22), and multidrug resistant strains have emerged. The ACSSuT (ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline) pattern of resistance, for instance, has been reported in multiple NTS serovars in different geographical regions (23-26). The emergence of widespread resistance in *Salmonella* is attributed to the extensive use of antibiotics in both clinical and agricultural settings (27-29), thereby reducing effectiveness of the commonly used antibiotics for therapy.

Michigan is not included in the FoodNet surveillance network, which monitors the incidence of foodborne illnesses and collects case information associated with these illnesses in the U.S. Consequently, this study was performed to determine the disease burden attributable to NTS infections in Michigan via active surveillance over a 4-year period and identify risk factors

for infection. We also sought to quantify the frequency of antibiotic resistance in NTS isolates and identify factors associated with resistance. This study highlights the importance of enhanced surveillance for resistant pathogens to ensure that the most appropriate drug targets are used, and to identify risk factors for infection and patients with an increased risk of more debilitating conditions.

MATERIALS AND METHODS

Strain source and collection

From 2011 to 2014, 198 NTS isolates were collected as part of the Enterics Research Investigational Network (ERIN) surveillance system, which was set up in collaboration with the Michigan Department of Health and Human Services (MDHHS) and four major hospitals in Michigan (Sparrow Health, University of Michigan Hospital, Spectrum Health and Detroit Medical Center). Isolates were cultured in Luria-Bertani (BD Diagnostics) media at 37^{o}C under aerobic conditions for 18-20h and were stored in Luria-Bertani broth with 10% glycerol at -80°C until further testing. All protocols used in this study were previously approved by the Institutional Review Boards at Michigan State University (MSU; Lansing, MI, USA; IRB #10-736SM) and the MDHHS (842-PHALAB) as well as each participating hospital.

Phenotypic antimicrobial susceptibility profiling

For NTS, susceptibilities to 24 antibiotics were determined by broth microdilution using Sensititre GN4F Trek plates (Trek Diagnostic Systems, Cleveland, OH, USA) according to the manufacturer's instructions. Eleven antibiotic classes were tested including aminoglycosides (amikcin, gentamicin, tobramycin), penicillins (ampicillin, piperacillin), β-lactam/β-lactamase inhibitor combination (ampicillin/sulbactam 2:1 ratio, pipercillin/tazobactam constant 4,

ticarcillin/clavulanic acid constant 2), cephalosporins (cefazolin, ceftazidime, ceftriaxone, cefipime), carbapenems (imipenem, doripenem, ertapenem, meropenem), tetracyclines (tetracycline, minocycline), fluoroquinolones (ciprofloxacin, levofloxacin), glycylcyclines (tigecycline), nitrofurans (nitrofurantoin), monobactams (aztreonam), and anti-folates (trimethoprim/sulfamethoxazole).. The Minimum Inhibitory Concentration (MIC) was determined by identifying the lowest concentration of antibiotic that prevented visible bacterial growth. *Escherichia coli* ATCC 25922, which is susceptible to all antibiotics evaluated, was used as the quality control strain. The results of the susceptibility tests were interpreted as resistant or susceptible in accordance with published guidelines for susceptibility testing (30). Isolates were defined as multidrug resistant if they were resistant to three or more classes of antimicrobial agents.

Data analysis

Epidemiological data and demographic data were obtained from the Michigan Disease Surveillance System (MDSS) and managed using Microsoft Access and Excel. Season was classified as spring (March, April, and May), summer (June, July, and August), fall (September, October, and November) and winter (December, January, and February) based on the sample collection date; for those cases with a missing collection date, the stool arrival date and/or onset dates were used. Counties in Michigan were classified as urban or rural based on the classification scheme devised by the National Center for Health Statistics (NCHS); only ten Michigan counties were classified as urban (31). Based on the published rates of antibiotic prescription and use in adults and children in Michigan (32), counties were classified as having high or low prescribing rates. High rates were classified as those counties where hospital service areas (HSAs) had >30% higher use relative to the state average. Dichotomous variables were created for length of hospital stay by defining long hospital stays as greater than the mean stay of four days. Cases using reverse

osmosis for water treatment were grouped with 'any well water consumption' since this treatment is likely used by consumers of well water.

SAS version 9.3 (SAS Institute, Cary, NC, USA) and Epi Info™ 7 were used for all statistical analyses. χ^2 test and Fisher's exact test were used for dichotomous variables to identify significant associations between the dependent and independent variables; a p value ≤ 0.05 was considered significant. A univariate analysis was first conducted, and those variables found to have strong associations with resistance (p value ≤ 0.20) were included in the multivariate analysis. Multivariate analysis using forward logistic regression was performed to build a model containing significant variables (p value ≤ 0.05) along with potentially confounding factors such as age and sex. The Mantel-Haenszel χ^2 test was used to test for trends while the student's t-test was used for testing statistical significance between means.

RESULTS

Characteristics of non-typhoidal *Salmonella* (NTS) cases in Michigan

A total of 198 clinical NTS isolates were recovered from 198 cases identified between January 2011 and December 2014 as part of the Michigan ERIN surveillance network (Table 6.1). Overall, 53.1% (n=104) of cases were male and 46.9% (n=92) were female, while most (n=82; 41.6%) cases were between 19 and 52 years of age. Patient sex and age were not known for two and one cases, respectively. When stratified by race, the proportion of cases differed significantly, with highest frequencies of infections occurring among Caucasians (n=125; 73.9%) compared to African Americans (n=33; 19.5%) and other races (n=11; 6.5%) (p<0.0001). The proportion of cases differed significantly among the four hospitals (p<0.0001), which could be due to variation

in surveillance activities across sites, yet no significant differences were observed by year (p=0.075).

Significantly higher frequencies of cases occurred in those patients who had not traveled in the past month (n=102, 61.8%) compared to those who did (n=63; 38.2%) (p<0.001). Additionally, a significant difference in proportion of cases was observed in patients who had contact with animals (n=94; 63.1%) relative to those who did not (n=55; 36.9%) (p=0.0014). Among the 94 cases with a history of animal contact, 13 (13.8%) had contact with reptiles such as turtles and lizards, and eight (8.5%) had contact with livestock including cattle, goats and pigs. Contact with domestic animals (e,g., cats, dogs and rabbits) was reported in 83 (88.3%) of the 94 cases with a history of animal contact. The most frequently reported symptoms were diarrhea (n=172, 97.7%) and abdominal pain (n=130, 78.8%) followed by fever (n=106, 69.3%), and 65 patients (34.6%) were hospitalized for a duration ranging between 1 day and 17 days; the average duration of hospitalization was four days.

When stratified by county, 55.9% (n=108) of the cases lived in rural counties, while 44.0% (n=85) were in urban counties; residence was not known for one case. Four cases resided in other states (Colorado, Georgia, Ohio and South Dakota), though each developed symptoms and were diagnosed with salmonellosis while in Michigan. We conducted a case-case analysis between rural and urban cases to determine differences in proportions between variables when stratified by residence (Table 6.2). The four cases residing in other states were excluded. The analysis indicated that animal contact was significantly more common in patients living in rural counties (OR: 2.1; 95% CI: 1.05-4.06); this included contact with birds (OR: 3.7; 95% CI: 1.02-13.39) and other animals (OR: 5.4; 95% CI: 1.51-19.11). Although the frequency of drinking well water was higher in rural counties (n=17, 18.9%) than urban (n=7, 11.7%), this difference was not significant.

Additionally, chicken consumption was lower in rural areas compared to urban areas (OR: 0.2; 95% CI: 0.06-0.84).

Risk factors associated with hospitalization due to NTS infections

To identify predictors of hospitalization, a marker for more severe infections, we conducted both univariate (Table 6.3) and multivariate logistic regression (Table 6.4) analyses using hospitalization as the dependent variable. Patients self-reporting nausea (OR:2.0; 95% CI: 1.03-3.93) and vomiting (OR: 2.0; 95% CI: 1.03-3.72) were significantly more likely to be hospitalized. The frequency of hospitalization was also highest for patients over the age of 60 years (n=15, 50.0%) when compared to patients younger than 10 years of age (n=14, 29.8%) and patients between 11 and 59 years (n=36; 32.4%). Moreover, patients from urban counties were more likely to be hospitalized (n=34; 41.5%) than those from rural areas (n=30; 28.8%). Multivariate logistic regression (Table 6.4) identified urban residence (OR: 2.4; 95% CI: 1.17-5.05) and nausea (OR: 2.2; 95% CI: 1.02-4.59) to be the predictors of hospitalization with NTS infections while controlling for age and sex.

Distribution of *Salmonella enterica* serovars in Michigan cases

The 198 NTS isolates were classified in to 35 different *S. enterica* serovars; the serovar could not be determined for three isolates. Among the 195 typed isolates, the predominant serovar was Enteritidis (n=72; 36.9%) followed by Typhimurium (n=38 ;19.5%), Newport (n=19; 9.7%), Hartford (n=6; 3.1%), Saintpaul (n=5; 2.6%) and Heidelberg (n=4; 2.1%). The remaining 51 isolates represented 28 different serovars with fewer than three isolates per type. Moreover, a subset of nine isolates were classified as I 4, [5], 12:i:- /I 4,5,12:i- (n=3), I 4, 12:b- (n=3), I 4, 12:i:- (n=2), and III 50:Kz (n=1) even though they are likely variants of known serovars.

A significant difference in the proportion of *S.* Enteritidis cases by hospital (p=0.02) was observed with highest frequencies of cases observed in Detroit Medical Center (n=24; 33.3%) and Sparrow Hospital (n=25; 34.7%). Additionally, proportion of *S.* Typhimurium cases also differed among the four hospitals (p=0.003), with highest frequencies of cases in University of Michigan Hospital (n=17; 44.7%) and Sparrow Hospital (n=13; 34.2%). Significant differences in the proportion of Newport cases (p=0.009) and other cases (p=0.0003) by hospital were also observed; one *S.* Heidelberg case was reported per hospital.

When stratified by serovar, no significant differences in the proportion of cases was observed each year. However, when stratified by year, significant differences in the proportion of serovars was observed. In 2011, high proportions of Enteritidis (n=12; 21.0%) and other serovars (n=28; 49.1%) were observed, followed by Typhimurium (n=9; 15.8%), Newport (n=7; 12.3%) and Heidelberg (n=1; 1.7%) (p<0.0001). A similar trend was also observed in 2014, with the highest frequencies of Enteritidis and other serovar cases (p<0.001). No Heidelberg cases were observed in 2012; while in 2013, only Enteritidis (n=20; 58.8%), Typhimurium (n=12; 35.3%) and Newport (n=2; 5.9%) cases were observed (p=0.0008).

The number of *S. enterica* cases also differed significantly by season, with the highest frequency of cases occurring in summer months (n=97; 48.9%) (p<0.0001). This trend was also observed when cases were stratified by serovars; in summer months, the frequencies of Enteritidis (n=34; 47.2%), Typhimurium (n=16, 42.1%), Newport (n=14; 73.8%), Heidelberg (n=2; 50%) and remaining serovars (n=29, 46.8%) were higher than in other months.

Furthermore, no associations between hospitalizations and serovars Enteritidis (n=21, 30.9%) and Typhimurium (n=11, 31.4%) were observed when all other serovars were grouped as the reference (n=31, 37.3%).

Risk factors for infection with specific *Salmonella* serovars

To identify the risk factors for different *Salmonella* serovars, a multinominal logit regression model (Table 6.7) was fit using data generated from the univariate analysis (Table 6.5 and Table 6.6). Several variables were found to be significant in the univariate analysis identifying risk factors of *S.* Enteritidis (Table 6.5). Higher frequencies of Enteritidis cases were observed in urban counties (n=40; 57.1%) compared to rural counties (n=31; 37.3%). Patients who consumed bottled water at home (n=11; 64.7%) were also significantly more likely to be infected by Enteritidis serovars than those who consumed municipal water (n=30; 37.0%). On the other hand, Typhimurium serovars were significantly associated with animal contact (Table 6.6), with higher frequencies of cases occurring in those patients who were exposed to animals (n=24; 38.1%) than those who were not (n=5; 15.1%). The univariate analysis also predicted contact with livestock (OR: 18.9; 95% CI: 2.21-162.26) and with other animals (OR: 3.1; 95% CI: 1.07-9.28) to be associated with Typhimurium infections. On further analysis, contact with either livestock or other animals is significantly associated with Typhimurium infections (OR: 4.4; 95% CI: 1.63-12.06) which is likely driving the association between any animal contact and Typhimurium infections. Thus, contact with any animal was used as the variable in the subsequent multinominal logit regression. For the multinominal logit regression, the outcome variable was infection with one of two serovars, Enteritidis or Typhimurium, while infection with the remaining serovars was used as the reference group. Although Newport and Heidelberg serovars are clinically important in the US, they were grouped in the reference group due to small sample size. Variables that were

associated with each outcome in the univariate analysis were included in the model as were potentially confounding factors such as age and sex. Variables that were found to have significant associations in Table 6.2 were also included in the multinominal logit regression analysis to control for residence. Drinking bottled water at home was found to be a predictor of both Enteritidis (OR: 6.1; 95% CI: 1.25-30.18) and Typhimurium infections (OR: 6.9; 95% CI: 1.23-39.29). Additionally, females were also more likely to be infected with Enteritidis serovars (OR: 2.9; 95% CI: 1.04-8.12). For acquiring Typhimurium infections, contact with animals approached statistical significance (OR: 3.5; 95% CI: 0.99-12.13; *p* value= 0.052).

Antibiotic resistance profiles of non-typhoidal *Salmonella* isolates

A high frequency of antibiotic resistance was observed, with 30 of the 198 (15.1%) NTS isolates showing resistance to at least one antibiotic (Figure 6.1). Resistance to ampicillin (11.6%) and tetracycline (11.1%) was most common followed by resistance to trimethoprim-sulfamethoxazole (2.5%), gentamicin (0.5%) and cephalosporins such as cefazolin (2.0%), ceftazidime (2.0%) and ceftriaxzone (1.0%). No resistance was observed to 13 out of the 24 antibiotics tested. Multidrug resistance to $\geq$ 3 antimicrobial classes was observed in 15 (7.5%) of the NTS isolates while four (2.0%) isolates were resistant to $\geq$ 4 antimicrobial classes; nine (4.5%) NTS isolates were resistant to only one antimicrobial class (Table 6.8). As shown in Figure 6.2, only ten serovars were observed to be resistant to $\geq$1 antibiotic. Additional, stratification by serovar revealed a significant difference in resistance frequencies between Enteritidis and all other NTS serovars (Fisher's exact *p* value<0.01). Furthermore, relative to *S.* Enteritidis, *S.* Typhimurium isolates are significantly more likely to be resistant (OR: 4.5; 95% CI: 1.27-16.22); four *S.* Enteritidis (n=72; 5.6%) and eight *S.* Typhimurium (n=38; 21.0%) isolates were resistant to at least one antibiotic (Figure 6.2).

Importantly, the proportion of all isolates resistant to at least one antibiotic increased over time, though the trend was not statistically significant (Maentel Hanzel *p* value= 0.077) (Figure 6.3). Interestingly, a significant increase in tetracycline resistance (*p* value <0.05), cephalosporin resistance (*p* value <0.05) and multidrug resistance (*p* value <0.05) was observed from 2011-2014. No significant differences in the frequency of resistance to trimethoprim-sulfamethoxazole and gentamicin were observed. A comparison between all NTS isolates (Figure 6.4A) from Michigan and those tested by the National Antimicrobial Resistance Monitoring System (NARMS) (33) revealed that resistance frequencies varied by antibiotic, although no significant differences were observed. For Enteritidis isolates, resistance to tetracycline was lower in Michigan isolates (n=1, 1.4%) than those tested by NARMS (n=48, 3.0%) (Figure 6.4B), although this difference was not significant. Additionally, frequency of resistance to ampicillin and tetracycline were higher in Typhimurium isolates tested by NARMS than in Michigan isolates (Figure 6.4C). However, resistance to trimethoprim-sulfamethoxazole was higher in Typhimurium isolates from Michigan (n=2, 5.3%) than isolates tested by NARMS (n=21, 1.7%), although this difference was not statistically significant.

Epidemiological associations with antibiotic resistant NTS infections

To identify factors associated with resistant NTS infections, we conducted univariate and multivariate analyses using resistance to at least one ($\geq$1) antibiotic as the dependent variable. The univariate analysis demonstrated that the odds of resistance was significantly higher in Typhimurium isolates (OR: 4.5; 95% CI: 1.27-16.22) and other NTS serovars (OR: 4.6; 95% CI: 1.47-14.20) compared to Enteritidis serovars (Table 6.9). Since the frequencies of resistance $\geq$1 antibiotic was similar for Typhimurium and other NTS serovars, they were grouped together for the subsequent multivariate analysis. Outbreak-associated strains were also 6.6 times (95% CI:

1.06-40.49) more likely to be resistant; however, the sample size was small and prevented inclusion in the multivariate analysis.

Higher resistance frequencies were also observed in counties with low antibiotic prescribing rates (n=26, 16.9%) compared to counties with high rates (n=4, 10.3%), although this difference was not statistically significant. Furthermore, frequencies of antibiotic resistant infections were higher in urban areas (n=15, 17.6%) than in rural areas (n=15, 13.9%). Variation in the frequency of resistant infections was also observed by season with the lowest frequencies occurring in the summer months (n=10, 10.3%) compared to winter, spring and fall (n=20, 19.8%) (OR: 2.1; 95% CI:0.95-4.86)).

Importantly, higher frequencies of antibiotic resistant infections were observed in hospitalized patients with longer hospital stays (n=6, 26.09%) compared to those patients with short hospital stays (n=5, 13.89%), however, no significant difference in these frequencies were observed. Multivariate analysis using forward regression indicated that all serovars other than Enteritidis were more likely to be resistant to at least one antibiotic (OR: 4.3; 95% CI: 1.18-15.62) than *S.* Enteritidis, and resistant NTS infections were also more likely to occur in winter, spring and fall (OR:3.5; 95% CI:1.23-9.72) than in summer.

DISCUSSION

In 2017, FoodNet reported the incidence of infection for *Salmonella* to be 16.0 per 100,000 people; the incidence rate for Enteritidis was 2.6 per 100,000 while for Typhimurium it was 1.4 per 100,000 (34). As an important food pathogen in the US, it is crucial to conduct continuous surveillance of NTS in order to form informed policies to reduce the disease burden. Since, Michigan is not one of the ten states included in the FoodNet surveillance network, our study is

one of the few that aims to determine factors associated with clinical NTS infections using a subset of NTS isolates recovered from clinical cases in Michigan. Additionally, this study is the first to determine antimicrobial resistance profiles and predictors of resistance in NTS using isolates collected from an active surveillance system in Michigan.

Interestingly, Younus et al. described the incidence and risk factors of *S.* Enteritidis infections using isolates from 1995-2001 collected by the Michigan Department of Community Health (35). This study identified children below the age of 4 years to be at a higher risk for *S.* Enteritidis infection. Thus, to build on this study and to determine risk factors for other NTS serovars, we conducted a multinominal logit regression to identify predictors of serovars Enteritidis and Typhimurium using isolates collected from the time-period 2011-2014. Our study identified drinking bottled water at home as a significant risk factor for acquiring both Enteritidis and Typhimurium infections. While drinking water from private wells that have been contaminated with human and animal feces (36) and unchlorinated ground water (37) have been associated with *Salmonella* infections, no study, to our knowledge, has identified bottled water as a risk factor of *Salmonella* infections. However, in 2016, Norovirus contamination of bottled water has been linked to an outbreak in Spain (38). Additionally, bottled water was also found to be a risk factor for *Campylobacter* infections in Cardiff, United Kingdom and has been suggested as a potential vehicle of transmission. People who drank bottled water and cold tap water were more likely to have been positive for *Campylobacter* than those who did not (39). Additionally, *Salmonella* spp. have also been isolated from bottled water in Bangladesh, having serious public health implications (40). While residence was not identified as significant predictor of NTS serovars in the multinominal logit regression, it is interesting to note that the frequency of Enteritidis cases were higher in urban areas compared to rural regions. Previous studies have documented a lower

prevalence of Enteritidis serovars compared to other serovars, such as Typhimurium, in the farm environment. Thomas et al. observed extremely low frequencies of *S.* Enteritidis isolated from tributaries located in rural areas in Canada (41). Additionally, low frequencies of *S.* Enteritidis were isolated from animals in Alberta (42); thus, providing some insights into whether other *Salmonella* serovars may be more widespread in the environment than Enteritidis. In addition, we found that the association between Typhimurium infections and animal contact approached statistical significance. Furthermore, we found that animal contact in rural areas is associated with NTS cases. Indeed, this observation has been recognized by many studies conducted worldwide. A study in Canada suggested that living on a livestock farm was an independent risk factor for acquiring *S.* Typhimurium DT104 infections (14). Furthermore, using phenotypic and genotypic methods, Hendriksen et al identified an indistinguishable *S.* Typhimurium DT104 isolate responsible for infecting a child and animals living on the same farm (43). The identification of animal contact as a factor strongly associated with NTS infections in Michigan is important as it highlights the need to practice strategies, such as frequent handwashing, aimed to prevent and control salmonellosis.

The incidence of antimicrobial drug resistant NTS in the US is estimated to be 1.93 per 100,000 person-years between 2004 and 2012 (44). Our study found high frequencies of resistance to antibiotics such as ampicillin and tetracycline. While tetracycline is not widely used in human medicine, it is an important antibiotic used routinely in veterinary medicine. The observation of tetracycline resistance NTS isolates in clinical cases may shed light on how antibiotic use in the farm environment may affect antibiotic resistance in clinical isolates. Indeed, antimicrobial drugs are widely used in food animals; an estimated 13×10^6 kilograms are used for therapeutic and sub-therapeutic purposes in animals in the US annually (45). Furthermore, many studies allude to the

association between antibiotic use in food animals and antibiotic resistance in humans. One such study reported finding identical antimicrobial resistance patterns in *E. coli* isolates from livestock and their farming families (46), highlighting the importance of elucidating the effects of agricultural antibiotic use on human health. While we did not detect any significant differences in frequencies between tetracycline resistant and susceptible cases that had animal contact as an exposure, it is important to consider that resistant bacteria from the farm environment can spread to humans through contamination of food products and water (47-51). Interestingly, we did not detect any resistance to ciprofloxacin among the NTS isolates. This is in accordance with several studies worldwide where extremely low frequencies to ciprofloxacin are reported (52, 53). In the US, no ciprofloxacin resistance was observed in NTS isolates from non-human sources (54). To prevent increasing frequencies of resistance to fluroquinolones, in 2005, the FDA prohibited the use of the fluoroquinolone enrofloxacin in poultry. Thus, to maintain low frequencies of resistance to fluroquinolones and employ these drugs as an important line of defense against NTS infections, it is imperative to practice judicious antibiotic use in both clinical and agricultural settings.

Additionally, our study also observed differences in frequencies of resistance based on serovars of NTS. Multivariate logistic regression identified serovar Enteritidis as a protective factor when resistance to at least one antibiotic was set as the dependent variable, with significantly higher frequencies of resistance observed in Typhimurium and other NTS serovars. Serovar dependent differences in resistance have been observed in NTS isolates in different geographic locations. Soler et al. found higher levels of resistance to serovars such as Typhimurium and Hadar than in Enteritidis isolates from Spain (53). Additionally, Voss-Rech et al. conducted a meta-analysis to determine the profile and temporal evolution of antibiotic resistant NTS in Brazil using research articles published between 1995 and 2014. They reported lower frequencies of antibiotic

resistance in *S.* Enteritidis compared to other NTS serovars in Brazil (55). While this significant difference in resistance levels between Enteritidis and other non-typhoidal serovars has not been explained yet, studies have attempted to explain this observation. Zhang et al. observed that ciprofloxacin resistant *S.* Typhimurium isolates were more competitive than ciprofloxacin resistant *S.* Enteritidis isolates (56). In addition, many studies have documented certain NTS serovars such as Kentucky, Typhimurium and Heidelberg to be multidrug resistant (57-59) while serovars such as Enteritidis, Montevideo, Infantis, and Mbandaka were observed to be pansusceptible or resistant to few antimicrobials (60, 61). Serovars such as Kentucky and Heidelberg have been shown to have a mutation in the methyl mismatch repair (MMR) system which may allowing for genetic heterogeneity (62) and this genome plasticity has been offered as an explanation for higher frequencies of antibiotic resistance (63). Thus, a difference in fitness between different serovars, genetic plasticity and dissimilar resistance mechanisms could explain the differing frequencies of resistance in NTS serovars worldwide. Furthermore, our study identified season as a predictor of antibiotic resistant infections, with more resistant infections occurring in fall, winter and spring. Prior studies have noted correlations between seasonality and antibiotic resistance frequencies. One such study observed higher frequencies of fluoroquinolone resistance in *Campylobacter* in winter and spring compared to summer; they attributed this difference to higher consumption of poultry products contaminated with resistant bacteria in the winter and more frequent exposure to susceptible *Campylobacter* through other sources during the summer months (64). In addition, our previous study also detected higher resistance frequencies in Shiga Toxin Producing *E. coli* (STEC) isolates in winter and spring compared to summer and fall (65). Interestingly, correlations between seasonal variations in antibiotic prescription and antibiotic resistance have been previously described (66, 67). These data suggest that many factors may influence this seasonal

variation in antibiotic resistance; however, further studies looking specifically at the state of Michigan are warranted to understand the factors driving antibiotic resistance in Michigan. CDC estimates that, in 2015, 918-1016 antibiotics per 1000 people were prescribed in Michigan (68). The high community antibiotic use, along with agricultural use of antibiotics, is likely a driving force behind high frequencies of antibiotic resistance in Michigan.

Prior studies have suggested that antibiotic resistant infections may be associated with severe disease outcomes such as mortality, hospitalizations and increased hospital stay (22, 65, 69, 70). Interestingly, in our study higher frequencies of antibiotic resistant isolates were isolated from patients that were hospitalized (n=12, 18.7%) compared to those who were not hospitalized (n=17, 13.8%), although this difference was not significant (*p* value=0.38) (data not shown). While we did not identify antibiotic resistant NTS infections to be a predictor of hospitalization, we did observe an association between antibiotic resistance in NTS and the length of hospitalization. Notably, the mean hospital stay was 5.91 days (n=11) for patients hospitalized with isolates resistant to at least one antibiotic compared to the mean hospital stay of 4.02 days when patients were infected with pansusceptible isolates (data not shown). Therefore, among the patients that were hospitalized, those who were infected with resistant NTS isolates had significantly longer hospital stays than those patients that were infected with pansusceptible isolates (Student's t-test *p* value <0.05). On comparison of cases between tetracycline resistant and tetracycline susceptible infections, the mean hospital stay was 4.15 days for patients hospitalized with tetracycline susceptible NTS infections, compared to a hospital stay of 6 days for patients infected with tetracycline resistant isolates (Student's t-test *p* value=0.068) (Table 6.10). Notably, the mean hospital stay was 6.2 days for patients infected with ampicillin resistant NTS infections, which was significantly longer than the mean hospital stay of 4 days when patients were infected with

ampicillin susceptible isolates (Student's t-test p value <0.05) (Table 6.10). The longer length in hospital stay could be attributed to the fact that resistant infections may take longer to clear than susceptible infections when the patient is on antibiotic therapy. Furthermore, increased hospital stay could be due to the requirement for increased surgical interventions to control the infection (70); several studies have observed an increased need for surgery when patients are battling antibiotic resistant infections (69, 71). It is also important to note, however, that numerous factors such as pathogen factors, host factors and treatment options may play an important role in influencing severe disease outcomes such as hospitalizations and longer hospital stays (70). For instance, patient factors such as age, gender and underlying co-morbidities may affect disease outcomes. Indeed, Bogan et al. identified comorbidities to be important factors for in-hospital mortality due to carbapenem-resistant Enterobacteriaceae infections (72). Also, the presence of genes encoding virulence and antibiotic resistance on the same genetic element may also provide insight into the association between disease severity and antibiotic resistance. Indeed, Srisanga et al. detected positive associations between virulence and resistance genes such as *bla*$_{\text{PSE-1}}$/*orgA* and *sul1*/*tolC* in *S. enterica* isolates (73). It is interesting to note that using isolates from FoodNet and NARMS, Varma et al. determined the odds of hospitalization with bloodstream NTS infection is significantly higher in those patients infected with resistant isolates than those infected with pansusceptible strains (22). Since we did not have data on the invasiveness of Michigan NTS strains, we were unable to determine if the observation seen in Varma et al.'s study holds true for Michigan. Furthermore, it is important to consider that the genetic diversity of NTS isolates in different geographical locations may also play a role in determining virulence and resistance profiles. It is quite possible that certain phylogenetic lineages of NTS circulating in Michigan are more likely to be associated with antibiotic resistance and virulence; further studies are required

to gain insights into this hypothesis. We did, however, identify patient residence as a predictor of hospitalization. We found that patients living in urban areas are more likely to be hospitalized with NTS infections than their rural counterparts. This has also been observed by Salinas et al. in Mexico, as they noted that people living in rural areas were less likely to visit physicians and also less likely to have been hospitalized (74). They attributed this difference to the lower likelihood of rural residents having health coverage. Furthermore, financial limitations and absence of hospitals could serve as alternate explanations for this observation.

To add to the problem of resistance, the emergence of multidrug resistant NTS is a huge public health burden as it limits the repertoire of antibiotics that can be used to treat these infections. Many studies worldwide have documented the rise of MDR *Salmonella*. Antibiotic resistance frequencies as high as 57.4% were observed in clinical and zoonotic *S.* Typhimurium strains isolated in Malaysia (75). Furthermore, an alarming increase in the prevalence of multidrug resistant NTS isolates from 1995 (12.4%) to 2015 (27.3%) has been reported in Australia (76). In the US, MDR *Salmonella* infections decreased from 1996 (17%) to 2008 (9.5%); however, an increase in MDR frequency was observed in 2015 (12%) (54). In our study, over the four-year period, 7.5% NTS isolates were multidrug resistant; however, we noted an increasing trend in prevalence of multidrug resistant NTS from 2011-2014 in Michigan, even though no significant differences in the proportion of cases were observed each year. Prior studies have attributed an increase in MDR isolates to international travel and import of food products (76, 77); however, we did not find international travel to be associated with MDR infections (data not shown); although a larger sample size of isolates needs to be tested to make further conclusions. Taken together, these observations have important implications for the treatment and control of multidrug resistant infections and the formation of policies to reduce the prevalence of MDR in Michigan.

Although the isolates analyzed in this study were collected as part of the ERIN active surveillance system, it is important to note that gastrointestinal illnesses are severely under-reported since not all individuals with gastrointestinal illnesses will seek medical care and stool samples are collected for only a fraction of individuals who seek medical care. Thus, it is possible that true resistance frequencies for NTS may be different from our findings. However, to ensure that the ERIN surveillance network is representative of the enteric infections occurring in Michigan, we previously confirmed that the frequency of ERIN cases was similar to those identified throughout the state of Michigan between 2011 and 2014 (78).

Since NTS are important enteric pathogens, continuous surveillance and monitoring of NTS infections is warranted to reduce its immense health burden. Additionally, routine testing of antimicrobial susceptibilities and determination of resistance profiles is important in order to aid medical personnel and public health officials determine and modify course of treatment of NTS infections. Furthermore, elucidating the risk factors of NTS and resistant NTS infections may help in the development of disease management policies and antibiotic use standards in order to curb the spread of NTS infections. Thus, the overall goal of this study is to help in the development of diagnostic guidelines and targeted intervention strategies, thereby aiding in overall disease management.

APPENDIX

Table 6.1. Characteristics of non-typhoidal *Salmonella* (NTS) cases in Michigan (2011-2014)

Characteristic	No. of cases‡	Percentage (%) of cases
Demographic data		
Sex		
Male	104	53.1%
Female	92	46.9%
Age group (years)		
≤ 2	17	8.6%
3 – 10	32	16.2%
11 – 18	22	11.2%
19 – 52	82	41.6%
≥53	44	22.3%
Race		
Caucasian	125	73.9%
African American	33	19.5%
Other	11	6.5%
Residence (counties in Michigan)		
Clinton	11	5.7%
Ingham	40	20.7%
Livingston	6	3.1%
Macomb	3	1.5%
Oakland	18	9.3%
Washtenaw	23	11.9%
Wayne	38	19.7%
Others	54	27.9%
Hospital		
Detroit Medical Center	46	23.3%
Sparrow Hospital	73	36.9%
Spectrum Health	21	10.6%
University of Michigan Hospital	58	29.3%
Epidemiological data		
Travel		
No travel	102	61.8%
Domestic travel	44	27.3%
International travel	20	12.9%
Animal Contact		
Any animal	94	63.1%
Reptile	13	8.5%
Livestock	8	5.3%
Birds/poultry	18	11.8%
Domestic	83	54.6%
Others	22	15.3%
Food consumption		
Turkey	42	79.2%
Chicken	111	84.7%

Table 6.1 (cont'd)

	88	91.6%
Beef	78	90.7%
Pork	83	61.9%
Deli meat	52	88.1%
Raw fruits	104	75.4%
Raw leafy greens	88	90.7%
Raw vegetables		
Water at home		
Any well	24	16.0%
Any municipal	103	68.7%
Only bottled	22	14.7%
Filtered/ Reverse osmosis	1	0.7%
Clinical Outcomes		
Case hospitalization	65	34.6%
Abdominal pain	130	78.8%
Body ache	57	36.1%
Diarrhea	172	97.7%
Bloody diarrhea	70	43.7%
Chills	71	44.4%
Fatigue	84	52.2%
Headache	54	34.2%
Nausea	92	56.4%
Vomiting	65	40.6%
Fever	106	69.3%

The percentages are based on the number of cases for which information was available. Counts for sex, age group, race, water at home are mutually exclusive for each category; counts for travel, animal contact and food consumption are repeated across categories as they are reported.

‡ Total number of cases varies between variables due to the difference in missing data.

Table 6.2. Univariate analysis of risk factors in rural cases (n=108) compared to urban cases (n=85)

Variables	Rural		Urban		Univariate analysis: rural vs. urban		
	Total cases	%	Total cases	%	OR	95% CI†	*p* value‡
Food consumption							
Turkey	23/32	71.9%	19/21	90.5%	0.3	0.05-1.39	0.17
Chicken	63/80	78.7%	48/51	94.1%	0.2	0.06-0.84	0.02
Beef	49/56	87.5%	39/40	97.5%	0.2	0.02-1.52	0.13
Pork	44/51	86.3%	34/35	97.1%	0.2	0.02-1.57	0.13
Deli meat	46/78	58.9%	37/56	66.1%	0.7	0.36-1.51	0.40
Raw fruits	32/38	84.2%	20/21	95.2%	0.3	0.03-2.38	0.40
Raw leafy greens	63/83	75.9%	41/55	74.5%	1.1	0.49-2.36	0.86
Raw vegetables	49/56	87.5%	39/41	95.1%	0.3	0.07-1.83	0.29
Peanut butter	41/83	49.4%	18/49	36.7%	1.7	0.82-3.46	0.16
Animal contact							
Any animal contact	61/87	70.1%	33/62	53.2%	2.1	1.05-4.06	0.03
Reptiles	9/92	9.8%	4/60	6.7%	1.5	0.44-5.17	0.57
Livestock	8/92	8.7%	0/59	0.0%	Undefined	Undefined	0.02
Birds	15/92	16.3%	3/60	5.0%	3.7	1.02-13.39	0.04
Domestic animals	55/91	60.4%	28/61	45.9%	1.8	0.93-3.47	0.08
Other animals	19/85	22.3%	3/59	5.1%	5.4	1.51-19.11	0.004
Water source at home							
Well	17/90	18.9%	8/60	13.3%	1.5	0.61-3.77	0.37

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables ≤5 in at least one cells.

Table 6.3. Univariate analysis of the frequency of hospitalizations due to non-typhoidal *Salmonella* (NTS) infections among patients in Michigan, 2011-2014

Characteristic	Total strains*	No (%) hospitalized	OR (95% CI)†	*p* value‡
Outbreak associated				
Yes	7	2 (28.6%)	0.9 (0.16-5.84)	1.0
No	38	11 (28.9%)	1.0	
Sex				
Male	98	35 (35.7%)	1.0	-
Female	89	30 (33.7%)	0.9 (0.50-1.67)	0.77
Age in years				
0-10	47	14 (29.8%)	0.4 (0.16-1.09)	0.07
11-59	111	36 (32.4%)	0.5 (0.21-1.09)	0.07
≥ 60	30	15 (50%)	1.0	-
Patient race				
Caucasian	122	44 (36.1%)	0.7 (0.34-1.36)	0.27
Other	44	20 (45.4%)	1.0	-
Residence				
Urban	82	34 (41.5%)	1.7 (0.95-3.22)	0.07
Rural	104	30 (28.8%)	1.0	
Abdominal pain				
Yes	130	48 (36.9%)	1.5 (0.65-3.31)	0.36
No	35	10 (28.6%)	1.0	-
Body ache				
Yes	57	18 (31.6%)	0.8 (0.40-1.59)	0.52
No	101	37 (36.6%)	1.0	-
Bloody diarrhea				
Yes	70	28 (40.0%)	1.6 (0.85-3.18)	0.14
No	90	26 (28.9%)	1.0	-
Fatigue				
Yes	84	30 (35.7%)	1.1 (0.57-2.09)	0.79
No	77	26 (33.8%)	1.0	-
Headache				
Yes	54	19 (35.2%)	1.1 (0.54-2.13)	0.85
No	104	35 (33.6%)	1.0	-
Nausea				
Yes	92	39 (42.4%)	2.0 (1.03-3.93)	0.04
No	71	19 (26.8%)	1.0	-
Vomiting				
Yes	65	29 (44.6%)	1.9 (0.99-3.72)	0.049
No	95	28 (29.5%)	1.0	-
Fever				
Yes	105	40 (38.1%)	1.4 (0.69-3.04)	0.32
No	47	14 (29.8%)	1.0	-

* Depending on the variable examined, the number of isolates does not add up to the total (n=198) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

Table 6.3 (cont'd)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells.

Table 6.4. Multivariate logistic regression analysis of the frequency of hospitalizations due to non-typhoidal *Salmonella* (NTS) infections among patients in Michigan, 2011-2014

Characteristic	Multivariate analysis		
	OR	95% CI €	*p* value‡
Sex: Female	0.6	0.28-1.19	0.13
Age in years: ≥ 60	2.2	0.87-5.64	0.10
Residence: Urban	2.4	1.17-5.05	0.012
Bloody Diarrhea: Yes	2.2	1.00-4.65	0.13
Vomiting: Yes	1.4	0.64-2.99	0.41
Nausea: Yes	2.2	1.02-4.59	0.029

£ Logistic regression was performed using forward selection while controlling for variables that yielded significant (P≤0.05) and strong (P≤0.20) associations with hospitalization in the univariate analysis (Table S2). Hosmer and Lemeshow Goodness-of-Fit test (*p*= 0.9561). All variables were tested for collinearity by analyzing the Eigen values and condition numbers.

€ Wald 95% confidence intervals (CI)

Table 6.5. Univariate analysis to identify risk factors for *Salmonella enterica* serovar Enteritidis infections

Characteristic	Total strains*	No (%) Enteritidis	OR (95% CI†)	*p* value‡
Pathogen factors				
Outbreak associated				
Yes	7	0 (0.0%)	Undefined	0.014
No	31	16 (51.6%)	1.0	-
Demographics factors				
Residence				
Urban	70	40 (57.1%)	2.2 (1.17-4.28)	0.014
Rural	83	31 (37.3%)	1.0	-
Age in years				
0-10	37	15 (40.5%)	0.7 (0.27-1.99)	0.54
11-59	93	44 (47.3%)	0.9 (0.41-2.28)	0.94
≥ 60	27	13 (48.1%)	1.0	-
Sex				
Male	82	37 (45.2%)	1.0	-
Female	74	35 (47.3%)	1.1 (0.58-2.05)	0.78
Race				
Caucasian	92	39 (42.4%)	0.6 (0.27-1.21)	0.14
Other	41	23 (56.1%)	1.0	-
Epidemiological factors				
Season				
Winter, Spring, Fall	78	38 (48.7%)	1.0	0.47
Summer	79	34 (43.0%)	0.8 (0.42-1.49)	-
Domestic travel in the past month				
Yes	36	11 (30.6%)	0.4 (0.19-1.02)	0.053
No	91	45 (49.4%)	1.0	-
Animal contact				
Yes	70	31 (44.3%)	1.1 (0.53-2.34)	0.78
No	48	20 (41.7%)	1.0	-
Livestock contact				
Yes	1	0 (0.0%)	Undefined	1.0
No	117	52 (44.4%)		-
Other animal contact				
Yes	13	5 (38.5%)	0.8 (0.24-2.60)	0.77
No	100	44 (44.0%)	1.0	-
Domestic animal contact				
Yes	62	24 (38.7%)	0.7 (0.33-1.39)	0.29
No	58	28 (48.3%)	1.0	-
Poultry consumption				
Yes	88	40 (45.4%)	5.0 (0.58-43.28)	0.13
No	7	1 (14.3%)	1.0	-
Chicken consumption				
Yes	83	37 (44.6%)	2.1 (0.68-6.40)	0.29
No	18	5 (27.8%)	1.0	-
Beef and pork consumption				
Yes	86	40 (46.5%)	5.2 (0.60-45.19)	0.12
No	7	1 (14.3%)	1.0	-

Table 6.5 (cont'd)

Water at home				
Any Municipal	81	30 (37.0%)	1.0	-
Any Well	19	10 (52.6%)	1.9 (0.69-5.17)	0.21
Bottled	17	11 (64.7%)	3.1 (1.04-9.29)	0.03

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells.

Table 6.6. Univariate analysis to identify risk factors for *Salmonella enterica* serovar Typhimurium infections

Characteristic	Total strains*	No (%) Typhimurium	OR (95% CI†)	*p* value‡
Pathogen factors				
Outbreak associated				
Yes	7	0 (0.0%)	Undefined	0.14
No	23	8 (34.8%)	1.0	-
Demographics factors				
Residence				
Urban	43	13 (30.2%)	0.9 (0.40-2.02)	0.80
Rural	77	25 (32.5%)	1.0	-
Age in years				
0-10	33	11 (33.3%)	2.3 (0.55-9.87)	0.33
11-59	73	24 (32.9%)	2.3 (0.59-8.72)	0.26
≥ 60	17	3 (17.6%)	1.0	-
Sex				
Male	65	20 (30.8%)	1.0	-
Female	56	17 (30.4%)	0.9 (0.45-2.13)	0.96
Race				
Caucasian	84	31 (36.9%)	3.5 (0.96-12.88)	0.067
Other	21	3 (14.3%)	1.0	-
Epidemiological factors				
Season				
Winter, Spring, Fall	62	22 (35.5%)	1.0	0.27
Summer	61	16 (26.2%)	0.6 (0.29-1.39)	-
Domestic travel in the past month				
Yes	33	8 (24.2%)	0.6 (0.24-1.56)	0.30
No	70	24 (34.3%)	1.0	-
Animal contact				
Yes	63	24 (38.1%)	3.4 (1.17-10.14)	0.02
No	33	5 (15.1%)	1.0	-
Livestock contact				
Yes	8	7 (87.5%)	18.9 (2.21-162.26)	0.0013
No	89	24 (26.9%)	1.0	-
Other animal contact				
Yes	17	9 (52.9%)	3.1 (1.07-9.28)	0.032
No	76	20 (26.3%)	1.0	-
Domestic animal contact				
Yes	59	21 (35.6%)	1.8 (0.74-4.60)	0.19
No	39	9 (23.1%)	-	-
Poultry consumption				
Yes	74	26 (35.1%)	3.2 (0.37-28.47)	0.41
No	7	1 (14.3%)	1.0	-
Chicken consumption				
Yes	72	26 (36.1%)	3.7 (0.77-17.56)	0.13
No	15	2 (13.3%)	1.0	-
Beef and pork consumption				
Yes	68	22 (32.3%)	2.87 (0.32-25.31)	0.43
No	7	1 (14.3%)	1.0	-

Table 6.6 (cont'd)

Water at home				
Any Municipal	71	20 (28.2%)	1.0	-
Any Well	15	6 (40.0%)	1.7 (0.53-5.39)	0.36
Bottled	11	5 (45.4%)	2.1 (0.58-7.75)	0.29

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables $\leq$5 in at least one cells.

Table 6.7. Multinominal logistic regression to identify the risk factors of *Salmonella* enterica serovar Enteritidis and *Salmonella* enterica serovar Typhimurium infections in Michigan, 2011-2014

Characteristic, by serovar	Multinominal Logistic Regression£		
	Odds Ratio	95% CI€	*p* value‡
Enteritidis			
Sex: Female	2.9	1.04-8.12	0.04
Age in years: ≥60	0.6	0.18-2.33	0.51
Patient Race: Caucasian	0.4	0.15-1.41	0.17
Residence: Urban	1.6	0.59-4.69	0.34
Domestic travel: Yes	0.5	0.16-1.54	0.23
Animal contact: Yes	1.8	0.63-4.96	0.28
Water at home: Bottled	6.1	1.25-30.18	0.02
Chicken consumption: Yes	1.6	0.36-7.07	0.53
Typhimurium			
Sex: Female	1.6	0.52-5.22	0.39
Age in years: ≥60	0.2	0.03-1.03	0.054
Patient Race: Caucasian	3.6	0.62-20.79	0.15
Residence: Urban	1.1	0.34-3.48	0.88
Domestic travel: Yes	0.5	0.12-1.38	0.15
Animal contact: Yes	3.5	0.99-12.13	0.052
Water at home: Bottled	6.9	1.23-39.29	0.03
Chicken consumption: Yes	3.7	0.59-22.78	0.16

£ Multinominal logistic regression was performed using variables found to have significant (P≤0.05) and strong (P≤0.20) associations with the serovars Enteritidis and Typhimurium in the univariate analysis (Tables S4 and S5). Confounding factors such as age and sex were also included in the analysis. All models used 'all other *Salmonella* serovars' as reference group. The likelihood ratio chi square value of 30.89 with a *p* value of 0.014 indicates that the variables fit in the model significantly better than a null model (likelihood ratio chi sq=2.31, *p* value=0.68)

€ Wald 95% confidence intervals (CI)

Table 6.8. Multidrug resistance in non-typhoidal *Salmonella* (NTS) in Michigan (2011-2014)

Resistance Pattern	No. (%) of Resistant Strains (n=198)
No resistance detected	168 (84.84%)
Resistance to 1 antimicrobial class	9 (4.5%)
Resistance to ≥2 antimicrobial class	21 (10.60%)
Resistance to ≥3 antimicrobial class	15 (7.5%)
Resistance to ≥4 antimicrobial class	4 (2.02%)

Table 6.9. Univariate and multivariate analysis to identify factors associated with antibiotic resistance in 198 clinical non-typhoidal *Salmonella* (NTS) in Michigan, 2011-2014

Characteristic	Total strains*	No (%) ≥1 resistance	OR (95% CI†)	*p* value‡
Pathogen factors				
Serovar				
Enteritidis	72	4 (5.6%)	1.0	-
Typhimurium	38	8 (21.05%)	4.5 (1.27-16.22)	0.021
Other	85	18 (21.18%)	4.6 (1.47-14.20)	0.005
Outbreak associated				
Yes	7	3 (42.9%)	6.6 (1.06-40.49)	0.06
No	39	4 (10.3%)	1.0	-
Demographics factors				
Residence				
Urban	85	15 (17.6%)	1.3 (0.61-2.89)	0.47
Rural	108	15 (13.9%)	1.0	-
Age in years				
0-10	49	8 (16.3%)	1.8 (0.44-7.47)	0.51
11-59	117	19 (16.2%)	1.8 (0.49-6.56)	0.57
≥ 60	31	3 (9.7%)	1.0	-
Sex				
Male	104	17 (16.3%)	1.0	-
Female	92	12 (13.0%)	0.8 (0.34-1.71)	0.51
Race				
Caucasian	125	19 (15.2%)	1.4 (0.49-4.0)	0.62
Other	44	5 (11.4%)	1.0	-
Antibiotic Prescription rates by county	39	4 (10.3%)	0.6 (0.18-1.72)	0.46
High	154	26 (16.9%)	1.0	-
Low				
Epidemiological and other factors				
Length of hospital stay				
Short (1-4 days)	36	5 (13.89%)	1.0	-
Long (=>5 days)	23	6 (26.09%)	2.2 (0.58-8.24)	0.31
Season				
Fall	42	12 (28.57%)	3.5 (1.36-8.87)	0.0067
Winter	22	2 (9.09%)	0.9 (0.18-4.28)	1.0
Spring	37	6 (16.22%)	1.7 (0.56-5.02)	0.34
Summer	97	10 (10.31%)	1.0	-

Table 6.9 (cont'd)

Domestic travel in the past month				
Yes	44	9 (20.4%)	2.0 (0.81-5.22)	0.12
No	117	13 (11.1%)	1.0	-
Animal contact				
Yes	91	11 (12.1%)	0.6 (0.25-1.64)	0.35
No	57	10 (17.5%)	1.0	-
Poultry consumption				
Yes	116	15 (12.9%)	1.0 (0.12-9.05)	1.0
No	8	1 (12.5%)	1.0	-
Beef and pork consumption				
Yes	110	18 (16.4%)	1.4 (0.16-11.82)	1.0
No	8	1 (12.5%)	1.0	-
Water at home				
Any municipal	103	14 (13.6%)	1.0	-
Any well	25	5 (20.0%)	1.6 (0.51-4.92)	0.53
Only bottled	22	4 (18.2%)	1.4 (0.42-4.79)	0.52

Characteristic	Multivariate Analysis		
	OR	**95% CI €**	***p* value‡**
Sex: Female	1.0	0.39-2.66	0.97
Age in years: ≥ 60	0.7	0.15-0.69	0.69
Serovar: Serovars excluding Enteritidis	4.3	1.18-15.62	0.02
Season: Winter, Fall and Spring	3.5	1.23-9.72	0.018
Domestic travel: Yes	1.9	0.74-5.24	0.17

* Depending on the variable examined, the number of isolates does not add up to the total (n=198) because of missing data.

† 95% confidence interval (CI) for odds ratio (OR)

‡ *p* value was calculated by Chi-square test and Fisher's exact test was used for variables ≤5 in at least one cells.

£ Logistic regression was performed using forward selection while controlling for variables that yielded significant (P≤0.05) and strong (P≤0.20) associations with hospitalization in the univariate analysis. The variable outbreak associated cases was not included in the multivariate

Table 6.9 (cont'd)

analysis due to the high number of missing data. Hosmer and Lemeshow Goodness-of-Fit test (P= 0.8353). All variables were tested for collinearity.

€ Wald 95% confidence intervals (CI)

Table 6.10. Characteristic of cases with resistant and susceptible non-typhoidal *Salmonella* (NTS) infections in Michigan, 2011-2014

Variable	No. (%) cases*	Tetracycline			Ampicillin		
		No (%) TET^R	No (%) TET^S	*p* value‡	No (%) AMP^R	No (%) AMP^S	*p* value‡
Rural residence	108 (54.8%)	12 (11.1%)	96 (88.9%)	0.69	12 (12.9%)	96 (88.9%)	0.69
Animal contact	91 (61.5%)	9 (9.9%)	82 (90.1%)	0.9	9 (9.9%)	82 (90.1%)	0.44
International travel (past month)	20 (12.9%)	2 (10.0%)	18 (90.0%)	1.0	3 (15.0%)	17 (85.0%)	0.71
Domestic travel (past month)	44 (27.3%)	6 (13.6%)	38 (86.4%)	0.25	5 (11.4%)	39 (88.6%)	1.0
Hospitalization	64 (34.2%)	7 (10.9%)	57 (89.1%)	0.93	10 (15.6%)	54 (84.4%)	0.24
Mean days in hospital	4 (n=59)	6 (n=7)	4.15 (n=52)	0.068†	6.2 (n=10)	4 (n=49)	0.0107†
Abdominal pain	130 (78.8%)	15 (11.5%)	115 (88.5%)	0.53	14 (10.8%)	116 (89.2%)	0.30
Body ache	57 (36.1%)	5 (8.8%)	52 (91.2%)	1.0	6 (10.5%)	51 (89.5%)	0.79
Bloody diarrhea	70 (43.7%)	8 (11.4%)	62 (88.6%)	0.43	8 (11.4%)	62 (88.6%)	0.95
Chills	71 (44.4%)	7 (9.9%)	64 (90.1%)	0.85	8 (11.3%)	63 (88.7%)	0.99
Fatigue	84 (52.2%)	10 (11.9%)	74 (88.1%)	0.38	11 (13.1%)	73 (86.9%)	0.59
Headache	54 (34.2%)	6 (11.1%)	48 (88.9%)	0.62	5 (9.3%)	49 (90.7%)	0.61
Nausea	92 (56.4%)	8 (8.7%)	84 (91.3%)	0.41	10 (10.9%)	82 (89.1%)	0.53
Vomiting	65 (40.6%)	6 (9.2%)	59 (90.8%)	0.96	7 (10.8%)	58 (89.2%)	0.87
Fever	106 (69.3%)	11 (10.4%)	95 (89.6%)	1.0	12 (11.3%)	94 (88.7%)	1.0

Table 6.10 (cont'd)

*The % frequency reported from total cases and the number of cases that were available for each variable are specified.

‡From Chi-square test or Fisher's exact test († Student's t-test for independent means).

Figure 6.1. Antibiotic resistance frequencies in non-typhoidal *Salmonella* (NTS) strains in Michigan (n=198). Abbreviations: TIM2, Ticarcillin / clavulanic acid constant 2; TET, Tetracycline; MIN, Minocycline; SXT, Trimethoprim / sulfamethoxazole; PIP, Piperacillin; GEN, Gentamicin; FAZ, Cefazolin; TAZ, Ceftazidime; A/S2, Ampicillin / sulbactam 2:1 ratio; AMP, Ampicillin; AXO, Ceftriaxone

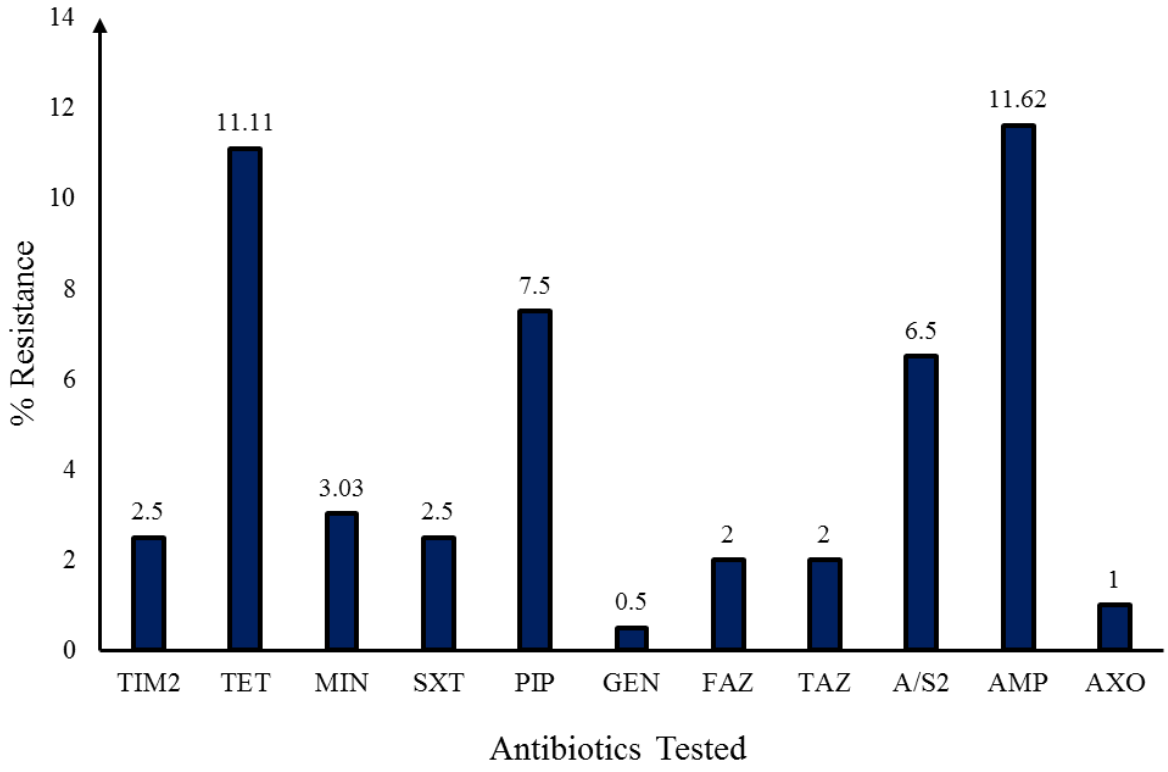


Figure 6.2. Frequency of antibiotic resistance in 195 clinical non-typhoidal *Salmonella* (NTS) isolates by serovar

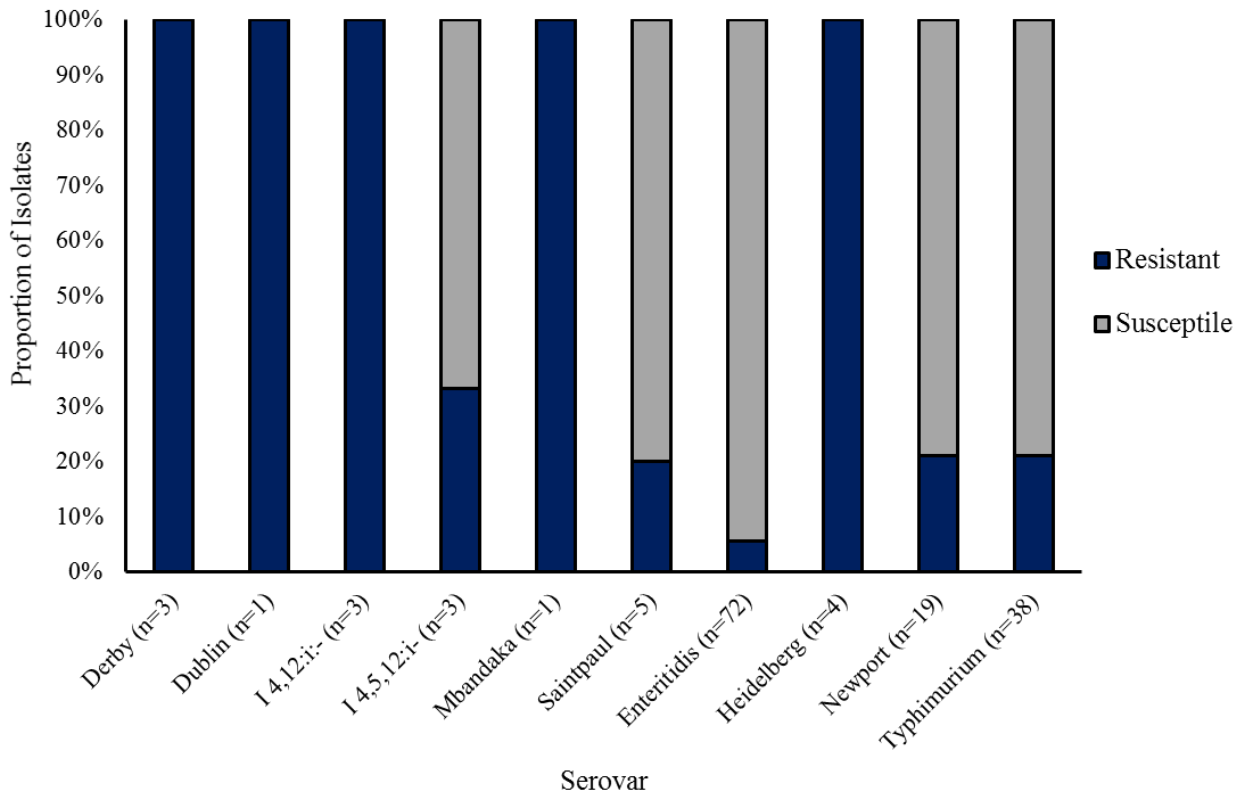
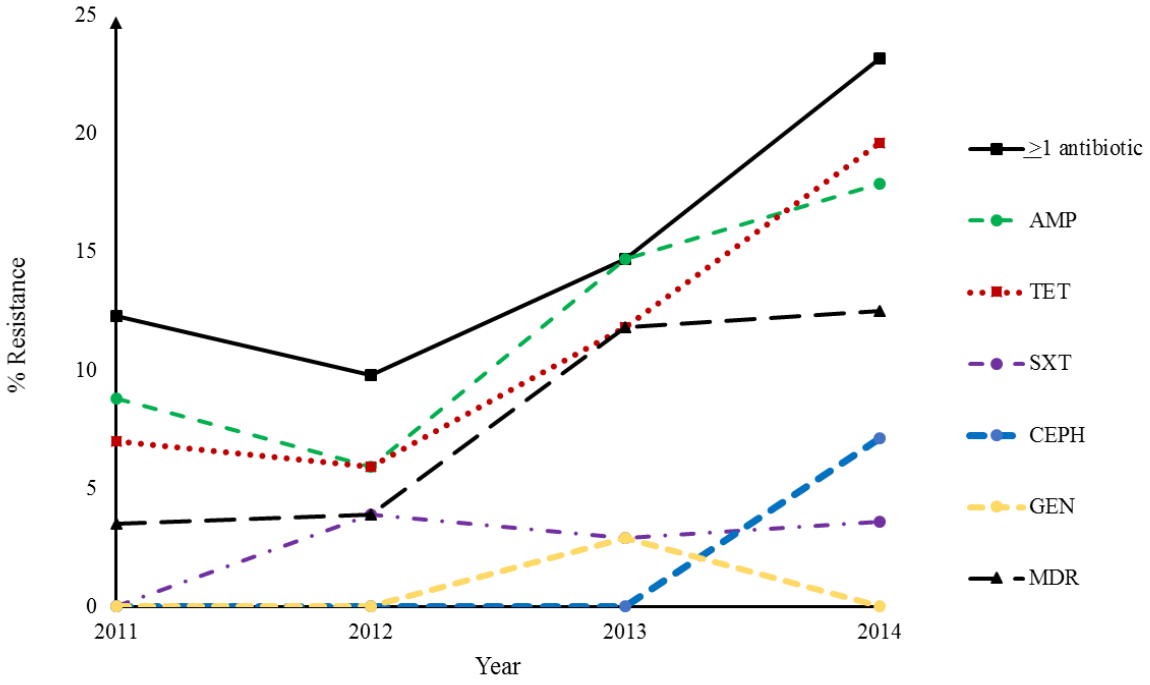


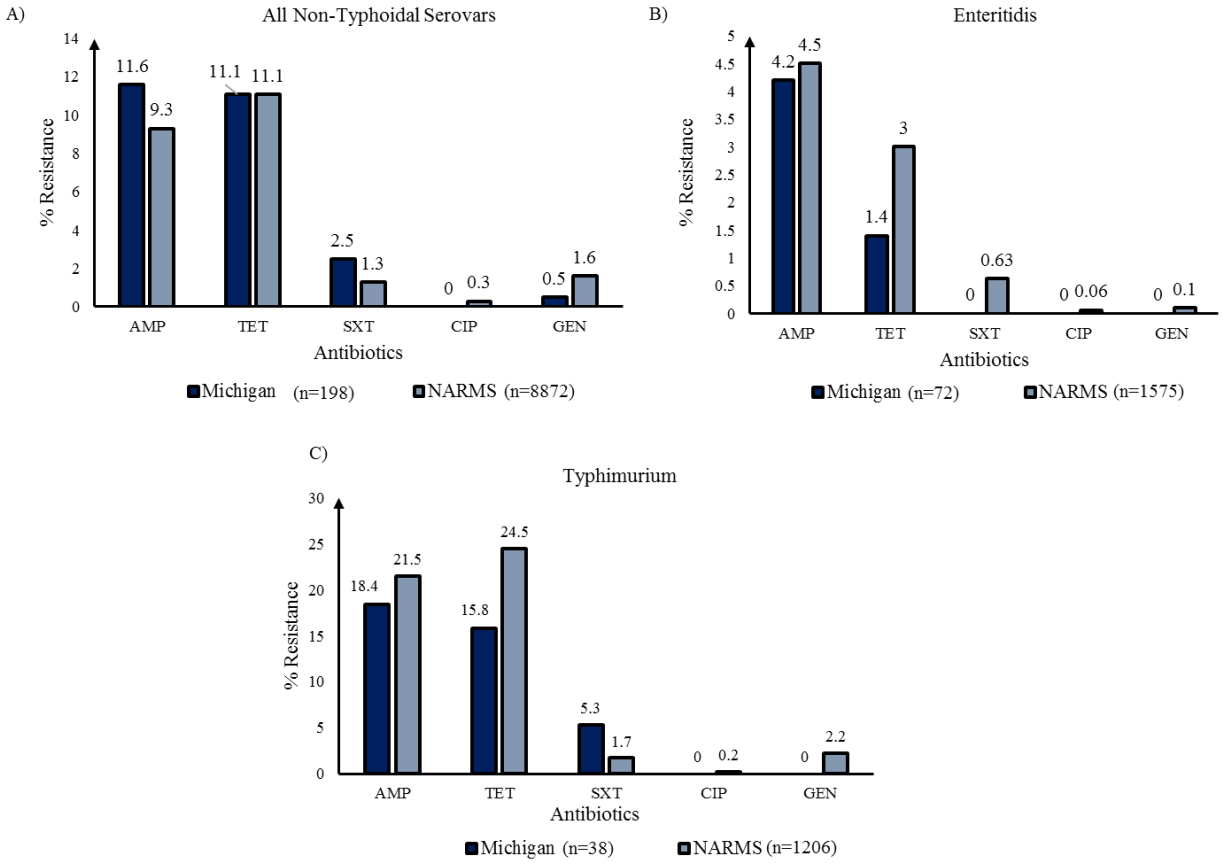
Figure 6.3. Trends in antimicrobial resistance over time observed in non-typhoidal *Salmonella* (NTS) isolates in Michigan, 2011-2014. Mantel-Haenszel chi-square was used to determine the trend and to calculate the *p* values. Abbreviations: AMP, Ampicillin; TET, Tetracycline; SXT, Trimethoprim / sulfamethoxazole; CEPH, Cephalosporin; GEN, Gentamicin; MDR, Multidrug resistance (resistance to ≥3 antimicrobial class)



% Resistance

Year	≥1 antibiotic	AMP	TET	SXT	CEPH	GEN	MDR
2011	12.3	8.8	7	0	0	0	3.5
2012	9.8	5.9	5.9	3.9	0	0	3.9
2013	14.7	14.7	11.8	2.9	0	2.9	11.8
2014	23.2	17.9	19.6	3.6	7.1	0	12.5
p-value	0.077	0.069	0.02	0.29	0.008	0.64	0.035

Figure 6.4. Frequency of resistance to various antimicrobials among non-typhoidal *Salmonella* (NTS) isolates in Michigan compared to those reported by the National Antimicrobial Resistance Monitoring System (NARMS) (79), 2011-2014. A) Resistance frequencies in all NTS serovars; B) Resistance frequencies in Enteritidis; C) Resistance frequencies in Typhimurium. Abbreviation: AMP, ampicillin; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; GEN, gentamicin



REFERENCES

REFERENCES

1. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM, International Collaboration on Enteric Disease 'Burden of Illness' Studies. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clin Infect Dis. 2010;50(6):882-9. doi: 10.1086/650733.

2. Global Burden of Diseases Diarrhoeal Diseases Collaborators. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. Lancet Infect Dis. 2017;17(9):909-48. doi: 10.1016/S1473-3099(17)30276-1.

3. Murray CJ, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet. 2012;380(9859):2197-223. doi: 10.1016/S0140-6736(12)61689-4.

4. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15. doi: 10.3201/eid1701.P1110110.3201/eid1701.091101p1.

5. Hoffmann S, Maculloch B, Batz M. Economic Burden of Major Foodborne Illnesses Acquired in the United States. Economic Information Bulletin 205081, United States Department of Agriculture, Economic Research Service. 2015.

6. Galanis E, Lo Fo Wong DM, Patrick ME, Binsztein N, Cieslik A, Chalermchikit T, Aidara-Kane A, Ellis A, Angulo FJ, Wegener HC for the World Health Organization Global Salm-Surv. Web-based surveillance and global *Salmonella* distribution, 2000-2002. Emerg Infect Dis. 2006;12(3):381-8. doi: 10.3201/eid1205.050854.

7. Crim SM, Iwamoto M, Huang JY, Griffin PM, Gilliss D, Cronquist AB, Cartter M, Tobin-D'Angelo M, Blythe D, Smith K, Lathrop S, Zansky S, Cieslak PR, Dunn J, Holt KG, Lance S, Tauxe R, Henao OL. Incidence and trends of infection with pathogens transmitted commonly through food- Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 2006-2013. MMWR Morb Mortal Wkly Rep. 2014;63(15):328-32.

8. Gal-Mor O, Boyle EC, Grassl GA. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. Front Microbiol. 2014;5:391. doi: 10.3389/fmicb.2014.00391.

9. Hoelzer K, Moreno Switt AI, Wiedmann M. Animal contact as a source of human non-typhoidal salmonellosis. Vet Res. 2011;42(1):34-. doi: 10.1186/1297-9716-42-34

10. Betancor L, Pereira M, Martinez A, Giossa G, Fookes M, Flores K, Barrios P, Repiso V, Vignoli R, Cordeiro N, Algorta G, Thomson N, Maskell D, Schelotto F, Chabalgoity JA.

Prevalence of *Salmonella enterica* in poultry and eggs in Uruguay during an epidemic due to *Salmonella enterica* serovar Enteritidis. J Clin Microbiol. 2010;48(7):2413-23. doi: 10.1128/JCM.02137-09.

11. Mermin J, Hutwagner L, Vugia D, Shallow S, Daily P, Bender J, Koehler J, Marcus R, Angulo FJ for the Emerging Infections Program FoodNet Working Group. Reptiles, amphibians, and human *Salmonella* infection: a population-based, case-control study. Clin Infect Dis. 2004;38 Suppl 3:S253-61. doi: 10.1086/381594.

12. Younus M, Wilkins MJ, Davies HD, Rahbar MH, Funk J, Nguyen C, Siddiqi AE, Cho S, Saeed AM. The role of exposures to animals and other risk factors in sporadic, non-typhoidal *Salmonella* infections in Michigan children. Zoonoses Public Health. 2010;57(7-8):e170-6. doi: 10.1111/j.1863-2378.2010.01324.x.

13. Doorduyn Y, Van Den Brandhof WE, Van Duynhoven YT, Wannet WJ, Van Pelt W. Risk factors for *Salmonella* Enteritidis and Typhimurium (DT104 and non-DT104) infections in The Netherlands: predominant roles for raw eggs in Enteritidis and sandboxes in Typhimurium infections. Epidemiol Infect. 2006;134(3):617-26. doi: 10.1017/S0950268805005406.

14. Dore K, Buxton J, Henry B, Pollari F, Middleton D, Fyfe M, Ahmed R, Michel P, King A, Tinga C, Wilson JB, Multi-Provincial *Salmonella* Typhimurium Case-Control Study Steering Committee. Risk factors for *Salmonella* Typhimurium DT104 and non-DT104 infection: a Canadian multi-provincial case-control study. Epidemiol Infect. 2004;132(3):485-93.

15. Baudart J, Lemarchand K, Brisabois A, Lebaron P. Diversity of *Salmonella* strains isolated from the aquatic environment as determined by serotyping and amplification of the ribosomal DNA spacer regions. Appl Environ Microbiol. 2000;66(4):1544-52.

16. Cherry WB, Hanks JB, Thomason BM, Murlin AM, Biddle JW, & , Croom JM. Salmonellae as an Index of Pollution of Surface Waters. Appl Microbiol. 1972;24(3):334-40.

17. Chao WL, Ding RJ, Chen RS. Survival of pathogenic bacteria in environmental microcosms. Zhonghua Min Guo Wei Sheng Wu Ji Mian Yi Xue Za Zhi [Chinese Journal of Microbiology and Immunology]. 1987;20(4):339-48.

18. Abdel-Monem MH, Dowidar A. Recoveries of *Salmonella* from soil in Eastern region of Saudi Arabia Kingdom. J Egypt Public Health Assoc. 1990;65(1-2):61-75.

19. Brigmon RL, Zam SG, Bitton G, Farrah SR. Detection of *Salmonella* Enteritidis in environmental samples by monoclonal antibody-based ELISA. J Immunol Methods. 1992;152(1):135-42.

20. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

21. Sanchez-Vargas FM, Abu-El-Haija MA, Gomez-Duarte OG. *Salmonella* infections: an update on epidemiology, management, and prevention. Travel Med Infect Dis. 2011;9(6):263-77. doi: 10.1016/j.tmaid.2011.11.001

22. Varma JK, Molbak K, Barrett TJ, Beebe JL, Jones TF, Rabatsky-Ehr T, Smith KE, Vugia DJ, Chang HG, Angulo FJ. Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. J Infect Dis. 2005;191(4):554-61. doi: 10.1086/427263.

23. Helms M, Ethelberg S, Mølbak K. International *Salmonella* Typhimurium DT104 infections, 1992-2001. Emerg Infect Dis. 2005;11(6):859-67. doi: 10.3201/eid1106.041017.

24. Yu CY, Chou SJ, Yeh CM, Chao MR, Huang KC, Chang YF, Chiou CS, Weill FX, Chiu CH, Chu CH, Chu C. Prevalence and characterization of multidrug-resistant (type ACSSuT) *Salmonella enterica* serovar Typhimurium strains in isolates from four gosling farms and a hatchery farm. J Clin Microbiol. 2008;46(2):522-6. doi: 10.1128/JCM.00709-07.

25. dos Reis EM, Rodrigues Ddos P, de Freitas-Almeida AC, Hofer E. Prevalence of R-type ACSSuT in strains of *Salmonella* serovar Typhimurium DT193 isolated from human infections in Brazil. Rev Panam Salud Publica. 2011;29(6):387-92.

26. Afema JA, Mather AE, Sischo WM. Antimicrobial Resistance Profiles and Diversity in *Salmonella* from Humans and Cattle, 2004-2011. Zoonoses Public Health. 2015;62(7):506-17. doi: 10.1111/zph.12172.

27. Emborg HD, Baggesen DL, Aarestrup FM. Ten years of antimicrobial susceptibility testing of *Salmonella* from Danish pig farms. J Antimicrob Chemother. 2008;62(2):360-3. doi: 10.1093/jac/dkn196.

28. Winokur PL, Brueggemann A, DeSalvo DL, Hoffmann L, Apley MD, Uhlenhopp EK, Pfaller MA, Doern GV. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob Agents Chemother. 2000;44(10):2777-83.

29. Gebreyes WA, Altier C. Molecular characterization of multidrug-resistant *Salmonella enterica* subsp. *enterica* serovar Typhimurium isolates from swine. J Clin Microbiol. 2002;40(8):2813-22.

30. Clinical & Laboratory Standards Insitute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Information Supplement. 2014.

31. Ingram DD, Franco SJ. 2013 NCHS Urban-Rural Classification Scheme for Counties. Vital Health Stat 2. 2014(166):1-73.

32. Kofke-Egger H, Udow-Phillips M. Antibiotic Prescribing and Use. Center for Healthcare Research and Transformation. 2011.

33. Centers for Disease Control and Prevention. Data from: National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. Atlanta, Georgia: U.S. Department of Health and Human Services, CDC. 2018.

34. Marder EP, Griffin PM, Cieslak PR, Dunn J, Hurd S, Jervis R, Lathrop S, Muse A, Ryan P, Smith K, Tobin-D'Angelo M, Vugia DJ, Holt KG, Wolpert BJ, Tauxe R, Geissler A. Preliminary Incidence and Trends of Infections with Pathogens Transmitted Commonly Through Food — Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2006–2017. MMWR Morb Mortal Wkly Rep 2018;67:324–8. doi: http://dx.doi.org/10.15585/mmwr.mm6711a3.

35. Younus M, Wilkins MJ, Arshad MM, Rahbar MH, Saeed AM. Demographic risk factors and incidence of *Salmonella* Enteritidis infection in Michigan. Foodborne Pathog Dis. 2006;3(3):266-73. doi: 10.1089/fpd.2006.3.266.

36. Centers for Disease Control and Prevention. *Salmonella* and Drinking Water from Private Wells. 2015.

37. Kovačić A, Huljev Ž, Sušić E. Ground water as the source of an outbreak of *Salmonella* Enteritidis. J Epidemiol Glob Health. 2017;7(3):181-4. doi: 10.1016/j.jegh.2017.05.001.

38. Blanco A, Guix S, Fuster N, Fuentes C, Bartolome R, Cornejo T, Pinto RM, Bosch A. Norovirus in Bottled Water Associated with Gastroenteritis Outbreak, Spain, 2016. Emerg Infect Dis. 2017;23(9):1531-4. doi: 10.3201/eid2309.161489.

39. Evans MR, Ribeiro CD, Salmon RL. Hazards of healthy living: bottled water and salad vegetables as risk factors for *Campylobacter* infection. Emerg Infect Dis. 2003;9(10):1219-25. doi: 10.3201/eid0910.020823.

40. Ahmed W, Yusuf R, Hasan I, Ashraf W, Goonetilleke A, Toze S, Gardner T. Fecal indicators and bacterial pathogens in bottled water from Dhaka, Bangladesh. Braz J Microbiol. 2013;44(1):97-103. doi: 10.1590/S1517-83822013005000026.

41. Thomas JL, Slawson RM, Taylor WD. *Salmonella* serotype diversity and seasonality in urban and rural streams. J Appl Microbiol. 2013;114(3):907-22. doi: 10.1111/jam.12079.

42. Guerin MT, Martin SW, Darlington GA, Rajic A. A temporal study of *Salmonella* serovars in animals in Alberta between 1990 and 2001. Can J Vet Res. 2005;69(2):88-99.

43. Hendriksen SW, Orsel K, Wagenaar JA, Miko A, van Duijkeren E. Animal-to-human transmission of *Salmonella* Typhimurium DT104A variant. Emerg Infect Dis. 2004;10(12):2225-7.

44. Medalla F, Gu W, Mahon BE, Judd M, Folster J, Griffin PM, Hoekstra RM. Estimated Incidence of Antimicrobial Drug-Resistant Nontyphoidal *Salmonella* Infections, United States, 2004-2012. Emerg Infect Dis. 2016;23(1):29-37. doi: 10.3201/eid2301.160771.

45. US Food and Drug Administration. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. 2011.

46. Fein D, Burton G, Tsutakawa R, Blenden D. Matching of antibiotic resistance patterns of *Escherichia coli* of farm families and their animals. J Infect Dis. 1974;130:274-9.

47. Mackie RI, Koike S, Krapac I, Chee-Sanford J, Maxwell S, Aminov RI. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. Anim Biotechnol. 2006;17(2):157-76. doi: 10.1080/10495390600956953.

48. Ejo M, Garedew L, Alebachew Z, Worku W. Prevalence and Antimicrobial Resistance of *Salmonella* Isolated from Animal-Origin Food Items in Gondar, Ethiopia. Biomed Res Int. 2016;2016:4290506. doi: 10.1155/2016/4290506.

49. White DG, Zhao S, Sudler R, Ayers S, Friedman S, Chen S, McDermott PF, McDermott S, Wagner DD, Meng J. The isolation of antibiotic-resistant *Salmonella* from retail ground meats. N Engl J Med. 2001;345(16):1147-54. doi: 10.1056/NEJMoa010315.

50. Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B, Nriagu J. Prevalence of antibiotic resistance in drinking water treatment and distribution systems. Appl Environ Microbiol. 2009;75(17):5714-8. doi: 10.1128/AEM.00382-09.

51. Ramteke PW, Gaur A, Pathak SP, Bhattacharjee JW. Antibiotic resistance of coliforms in drinking water in rural areas. Indian J Med Res. 1990;91:185-8.

52. Murray A, Coia JE, Mather H, Brown DJ. Ciprofloxacin resistance in non-typhoidal *Salmonella* serotypes in Scotland, 1993-2003. J Antimicrob Chemother. 2005;56(1):110-4. doi: 10.1093/jac/dki164.

53. Soler P, Gonzalez-Sanz R, Bleda MJ, Hernandez G, Echeita A, Usera MA. Antimicrobial resistance in non-typhoidal *Salmonella* from human sources, Spain, 2001-2003. J Antimicrob Chemother. 2006;58(2):310-4. doi: 10.1093/jac/dkl223.

54. National Antimicrobial Resistance Monitoring System. NARMS 2015 Integrated Report. 2015.

55. Voss-Rech D, Potter L, Vaz CS, Pereira DI, Sangioni LA, Vargas AC, de Avila Botton S. Antimicrobial Resistance in Nontyphoidal *Salmonella* Isolated from Human and Poultry-Related Samples in Brazil: 20-Year Meta-Analysis. Foodborne Pathog Dis. 2017;14(2):116-24. doi: 10.1089/fpd.2016.2228.

56. Zhang CZ, Ren SQ, Chang MX, Chen PX, Ding HZ, Jiang HX. Resistance mechanisms and fitness of *Salmonella* Typhimurium and *Salmonella* Enteritidis mutants evolved under selection with ciprofloxacin in vitro. Sci Rep. 2017;7(1):9113. doi: 10.1038/s41598-017-09151-y.

57. Roy P, Dhillon AS, Lauerman LH, Schaberg DM, Bandli D, Johnson S. Results of *Salmonella* isolation from poultry products, poultry, poultry environment, and other characteristics. Avian Dis. 2002;46(1):17-24. doi: 10.1637/0005-2086(2002)046[0017:ROSIFP]2.0.CO;2.

58. Berrang ME, Bailey JS, Altekruse SF, Shaw WK, Jr., Patel BL, Meinersmann RJ, Fedorka-Cray PJ. Prevalence, serotype, and antimicrobial resistance of *Salmonella* on broiler carcasses postpick and postchill in 20 U.S. processing plants. J Food Prot. 2009;72(8):1610-5.

59. Han J, David DE, Deck J, Lynne AM, Kaldhone P, Nayak R, Stefanova R, Foley SL. Comparison of *Salmonella enterica* serovar Heidelberg isolates from human patients with those from animal and food sources. J Clin Microbiol. 2011;49(3):1130-3. doi: 10.1128/JCM.01931-10.

60. Edrington TS, Schultz CL, Bischoff KM, Callaway TR, Looper ML, Genovese KJ, Jung YS, McReynolds JL, Anderson RC, Nisbet DJ. Antimicrobial resistance and serotype prevalence of *Salmonella* isolated from dairy cattle in the southwestern United States. Microb Drug Resist. 2004;10(1):51-6. doi: 10.1089/107662904323047808.

61. M'Ikanatha N M, Sandt CH, Localio AR, Tewari D, Rankin SC, Whichard JM, Altekruse SF, Lautenbach E, Folster JP, Russo A, Chiller TM, Reynolds SM, McDermott PF. Multidrug-resistant *Salmonella* isolates from retail chicken meat compared with human clinical isolates. Foodborne Pathog Dis. 2010;7(8):929-34. doi: 10.1089/fpd.2009.0499.

62. Dhanani AS, Block G, Dewar K, Forgetta V, Topp E, Beiko RG, Diarra MS. Genomic Comparison of Non-Typhoidal *Salmonella enterica* Serovars Typhimurium, Enteritidis, Heidelberg, Hadar and Kentucky Isolates from Broiler Chickens. PLoS One. 2015;10(6):e0128773. doi: 10.1371/journal.pone.0128773.

63. Shah DH, Paul NC, Sischo WC, Crespo R, Guard J. Population dynamics and antimicrobial resistance of the most prevalent poultry-associated *Salmonella* serotypes. Poult Sci. 2017;96(3):687-702. doi: 10.3382/ps/pew342.

64. Talsma E, Goettsch WG, Nieste HLJ, Schrijnemakers PM, Sprenger MJW. Resistance in *Campylobacter* species: increased resistance to fluoroquinolones and seasonal variation. Clin Infect Dis. 1999;29(4):845-8. doi: 10.1086/520447.

65. Mukherjee S, Mosci RE, Anderson CM, Snyder BA, Collins J, Rudrik JT, Manning SD. Antimicrobial Drug-Resistant Shiga Toxin-Producing *Escherichia coli* Infections, Michigan, USA. Emerg Infect Dis. 2017;23(9):1609-11. doi: 10.3201/eid2309.170523.

66. Sun L, Klein EY, Laxminarayan R. Seasonality and temporal correlation between community antibiotic use and resistance in the United States. Clin Infect Dis. 2012;55(5):687-94. doi: 10.1093/cid/cis509.

67. Suda KJ, Hicks LA, Roberts RM, Hunkler RJ, Taylor TH. Trends and seasonal variation in outpatient antibiotic prescription rates in the United States, 2006 to 2010. Antimicrob Agents Chemother. 2014;58(5):2763-6. doi: 10.1128/AAC.02239-13.

68. Centers for Disease Control and Prevention. Antibiotic Use in the United States, 2017: Progress and Opportunities. Atlanta, GA: US Department of Health and Human Services, CDC. 2017.

69. Linden PK, Pasculle AW, Manez R, Kramer DJ, Fung JJ, Pinna AD, Kusne S. Differences in outcomes for patients with bacteremia due to vancomycin-resistant *Enterococcus faecium* or vancomycin-susceptible *E. faecium*. Clin Infect Dis. 1996;22(4):663-70.

70. Cosgrove S. The Relationship between Antimicrobial Resistance and Patient Outcomes: Mortality, Length of Hospital Stay, and Health Care Costs. Clin Infect Dis. 2006;42(2):S82–S9. doi: https://doi.org/10.1086/499406

71. Carmeli Y, Eliopoulos G, Mozaffari E, Samore M. Health and economic outcomes of vancomycin-resistant enterococci. Arch Intern Med. 2002;162(19):2223-8.

72. Bogan C, Kaye KS, Chopra T, Hayakawa K, Pogue JM, Lephart PR, Bheemreddy S, Lazarovitch T, Zaidenstein R, Perez F, Bonomo RA, Marchaim D. Outcomes of carbapenem-resistant Enterobacteriaceae isolation: matched analysis. Am J Infect Control. 2014;42(6):612-20. doi: 10.1016/j.ajic.2014.02.013.

73. Srisanga S, Angkititrakul S, Sringam P, Le Ho PT, T Vo AT, Chuanchuen R. Phenotypic and genotypic antimicrobial resistance and virulence genes of *Salmonella enterica* isolated from pet dogs and cats. J Vet Sci. 2017;18(3):273-81. doi: 10.4142/jvs.2017.18.3.273.

74. Salinas JJ, Al Sinh S, Markides K, Ray LA, Angel RJ. The rural-urban divide: health services utilization among older Mexicans in Mexico. J Rural Health. 2010;26(4):333-41. doi: 10.1111/j.1748-0361.2010.00297.x.

75. Benacer D, Thong KL, Watanabe H, Puthucheary SD. Characterization of drug resistant *Salmonella enterica* serotype Typhimurium by antibiograms, plasmids, integrons, resistance genes and PFGE. J Microbiol Biotechnol. 2010;20(6):1042-52.

76. Williamson D, Lane C, Easton M, Valcanis M, Strachan J, Veitch M, Kirk M, Howden B. Increasing antimicrobial resistance in non-typhoidal *Salmonella* in Australia, 1979 - 2015 Antimicrob Agents Chemother. 2017. doi: doi:10.1128/AAC.02012-17.

77. Aarestrup FM, Hendriksen RS, Lockett J, Gay K, Teates K, McDermott PF, White DG, Hasman H, Sorensen G, Bangtrakulnonth A, Pornreongwong S, Pulsrikarn C, Angulo FJ, Gerner-Smidt P. International spread of multidrug-resistant *Salmonella* Schwarzengrund in food products. Emerg Infect Dis. 2007;13(5):726-31. doi: 10.3201/eid1305.061489.

78. Singh P, Teal TK, Marsh TL, Tiedje JM, Mosci R, Jernigan K, Zell A, Newton DW, Salimnia H, Lephart P, Sundin D, Khalife W, Britton RA, Rudrik JT, Manning SD. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome. 2015;3:45-. doi: 10.1186/s40168-015-0109-2.

79. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System (NARMS) Now: Human Data. 2018.

CHAPTER 7

Conclusions and Future Directions

Despite increased efforts to control the emergence and spread of antibiotic resistance, the global burden of antibiotic resistance infections is immense. Indeed, the 'Review on Antimicrobial Resistance' estimated that approximately 700,000 deaths worldwide are attributed to drug resistant infections alone (1). Importantly, this figure is expected to increase considerably by the year 2050, with approximately 10 million deaths projected to occur worldwide due to drug resistance (1). In the US, 2 million people acquire antibiotic resistant bacterial infections resulting in approximately 23,000 deaths every year (2). The CDC considers antibiotic resistance in numerous enteric pathogens such as non-typhoidal *Salmonella*, *Campylobacter* and *Shigella* to be of particular concern (2). To control the spread of antibiotic resistance in enteric pathogens, it is imperative to conduct continuous surveillance of resistance in both clinical and agricultural settings, identify risk factors of resistant infections to design targeted management and intervention strategies, and implement genomic tools to track and detect antibiotic resistance in a timely manner. Considering the importance of enteric pathogens such as Shiga toxin-producing *E. coli* (STEC) and non-typhoidal *Salmonella* (NTS) and the emergence of antibiotic resistance in these pathogens both in the US and worldwide, this study was undertaken to examine antibiotic resistance and factors associated with resistance in STEC and NTS isolates collected in Michigan.

The work in this dissertation offers insights into the overall frequencies of antibiotic resistance and increasing rates of resistance in STEC and NTS over large periods of time in Michigan. STEC and NTS isolates from patients were collected in collaboration with the Michigan Department of Health and Human Services and examined for resistance to clinically relevant antibiotics. In Chapter 3, which is a descriptive epidemiological study looking at STEC isolates from 2001-2014, we identified an increasing trend in antibiotic resistance, particularly to ampicillin and trimethoprim-sulfamethoxazole. Notably, significantly higher frequencies of

resistance were observed in 2010-2014 than in 2001-2009, indicating the emergence of antibiotic resistant STEC in recent years in Michigan. Similarly, as shown in Chapter 6, an increasing trend in ampicillin, tetracycline and multi-drug resistant NTS from 2011-2014 was also observed in Michigan. These findings highlight the importance of continuous monitoring of antibiotic resistance, worldwide. Surveillance of antibiotic resistance in a population is of paramount public health importance to guide the actions of clinicians, veterinarians and policy makers in the decision-making and action initiatives to combat the emergence of antibiotic resistance.

The negative impacts of antibiotic resistant infections on patient health outcomes have been well documented. According to the WHO, antibiotic resistant infections are more likely to result in severe disease outcomes, longer hospitalizations and higher risks of death compared to pansusceptible isolates (3). To this end, we sought to examine whether antibiotic resistant STEC and NTS in Michigan resulted in severe disease outcomes. Indeed, when compared to patients infected with pansusceptible NTS isolates, patients infected with antibiotic resistant NTS isolates had significantly longer mean hospital stays. Not only is this concerning due to the negative impact on patient health, but also due to the increased cost of patient care. Furthermore, in Chapter 2, we identified antibiotic resistant STEC to be independently associated with hospitalizations, which could indicate that antibiotic resistance resulted in more severe disease outcomes. This finding has important implications in the control of antibiotic resistant STEC, and additional variables such as hospitalization length, duration of illness etc. should be analyzed. Since our study is the first to identify this association in STEC, the examination of a larger set of STEC isolates from multiple geographical locations is warranted to determine if this finding is also observed in STEC isolates from other geographical regions. A matched case-control study, where hospitalized STEC cases are age, sex-matched with non-hospitalized STEC cases, may be an appropriate approach to

identify whether antibiotic resistant STEC infections are more likely to result in hospitalizations. Furthermore, it is also possible to identify mechanisms by which antibiotic resistant infections result in severe disease outcomes. For instance, whole genome sequencing of antibiotic resistant STEC strains may identify co-occurrence of resistance and virulence genes on the same mobile genetic element. The use of long-read sequencing (4) may aid in the identification of the precise location of resistance genes and virulence genes on mobile genetic elements using databases such as PlasmidFinder (5) or annotation of cassette and integron data (ACID) (6). Additionally, differences in disease severity and pathogenesis between antibiotic resistant and susceptible isolates can be identified using *in vitro* and *in vivo* models. For example, quantification of adherence and invasiveness of resistant and susceptible isolates in MAC-T bovine epithelial cells (7) or other cell lines (8) can be performed using association and invasive assays. The use of *in vivo* mouse models can also be used to study differences in pathogenicity by measuring morbidity, mortality and histopathological changes in the kidney and intestine (8) between resistant and susceptible STEC infections.

With differences observed in antibiotic resistance frequencies in serotypes of STEC and serovars of NTS, future work should involve further exploration into these serotype/serovar specific differences contributing to antibiotic resistance. One avenue of exploration could look at fitness differences between antibiotic resistant serotypes/serovars. As hypothesized in other studies, certain antibiotic resistant serotypes or serovars may have fitness benefits thus resulting in higher prevalence of certain serotypes/serovars (9). This could be achieved by conducting fitness experiments looking at differences in bacterial growth and bacterial competition between antibiotic resistant serotypes/serovars. Genome plasticity due to mutations in the methyl mismatch repair (MMR) system has also been offered as a possible explanation for differing antibiotic

resistance frequencies by serotype/serovars (10). Identification of such mutations in repair systems in isolates examined in this study may also shed light on serotype/serovars specific variations in antibiotic resistance.

There is a scarcity in information about genes conferring resistance to various antibiotics in STEC isolates and phylogenetic lineages of strains that may be associated with antibiotic resistance. However, the work shown here demonstrated a diversity in antibiotic resistance mechanisms and genes present in STEC isolates in Michigan. Most resistance genes were identified to be horizontally acquired, which is concerning due to ease of transmission of these genes to other strains belonging to the same or different pathogenic bacterial genera. Importantly, we also observed co-occurrence of multiple antibiotic resistance genes, likely on mobile genetic elements, highlighting the ease with which multi-drug resistant STEC strains can emerge in Michigan. We also examined the applicability of using whole genome sequencing for detection of antibiotic resistance. Overall, we observed high correlation between phenotypic and genotypic antibiotic susceptibility results. However, one STEC isolate that was phenotypically resistant to ampicillin was not identified to have any known antibiotic resistance genes. The use of other antibiotic resistance databases such as CARD and ARG-ANNOT or the use of gene prediction tools for identification of novel genes (11) may aid in the identification of the genes responsible for resistance in this isolate. Moreover, future studies should investigate the resistome of the human gut using samples collected from the ERIN study, which would in turn shed light on horizontal gene transfer of ARGs between commensal gut microorganisms and enteric pathogens such as STEC and NTS. Indeed, the importance of the human gut microbiome in the transfer of ARGs has been noted and the use patient resistome data to guide antibiotic therapy has been considered (12, 13).

Lastly, the work provided in this dissertation also determined antibiotic resistance frequencies in STEC isolated from cattle in Michigan (Chapter 5). Since the increasing importance of the intersection of human, animal and environmental health is being recognized (14, 15), we sought to employ this 'One Health' approach and integrate the sectors of human and animal health to study antibiotics resistance in STEC. The detection of the emergence and spread of antibiotic resistance in animal reservoirs is crucial to control the spread of resistance in clinically relevant pathogens, since many of these pathogens have animal reservoirs. High frequencies of resistance to both tetracycline and trimethoprim-sulfamethoxazole were observed; interestingly, resistance was only observed in beef herds and not dairy cattle. These findings warrant further investigations into herd and farm specific practices such as cleaning methods, disease prevention strategies, etc. Furthermore, determining the frequencies of antibiotic resistant STEC in environmental samples in proximity to these farms may also provide additional clues into transmission of antibiotic resistance. Since, little is known about the emergence of antibiotic resistant STEC and resistance genes in the cattle reservoir, identifying factors associated with resistant infections will help in the development of effective control strategies. Although unexplored in this dissertation, a comparative analysis between antibiotic resistant STEC isolates from humans and cattle may provide valuable information about transmission dynamics of antibiotic resistance between humans and cattle and shed light on antibiotic resistant phylogenetic lineages that are shared between humans and cattle. This can be accomplished by creating phylogenetic trees using seven loci MLST scheme from both cattle and human STEC isolates and examining the associations between phylogenies and antibiotic resistance using statistical methods. Interestingly, we did not observe any resistance to ampicillin in STEC isolates from cattle (Chapter 5), unlike what was observed for human derived isolates (Chapters 2 and 3), suggesting that different antibiotic

resistant STEC phylogenies may be circulating in the clinical and agricultural environment. Since in this study, samples from cattle were collected based on convenience, future studies could focus on sampling efforts applied proportionally according to the number of beef and dairy farms in counties, thus ensuring that cattle sampled are representative of the state of Michigan and antibiotic frequency data is generalizable.

Overall, high frequencies of antibiotic resistance were observed in STEC and NTS in Michigan. In addition to resulting in numerous foodborne illnesses worldwide, the widespread dissemination of antibiotic resistance in STEC and NTS is alarming as efficacy of treatment of bacterial illnesses in limited, and, in some instances, resulting in severe disease outcomes. As seen in our study, since many resistance genes were found to be those that are horizontally acquired, the acquisition of resistance genes by other clinically relevant pathogens is of great concern. A better understanding of the prevalence and risk factors of antibiotic resistant isolates from both humans and cattle will aid in disease management. The increasing rates of antibiotic resistance in both STEC and NTS in Michigan, call for the need of continuous surveillance to detect the emergence and spread of antibiotic resistance.

REFERENCES

REFERENCES

1. O'Neill J. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations. Review on Antimicrobial Resistance. 2014:1-16.

2. Centers for Disease Control and Prevention. Antibiotic resistance threats. 2013.

3. World Health Organization. Antimicrobial resistance: Global Report on Surveillance. 2014.

4. Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome assemblies with multiplex MinION sequencing. Microb Genom. 2017;3(10):e000132. doi: 10.1099/mgen.0.000132.

5. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 2014;58(7):3895-903. doi: 10.1128/AAC.02412-14.

6. Joss MJ, Koenig JE, Labbate M, Polz MF, Gillings MR, Stokes HW, Doolittle WF, Boucher Y. ACID: annotation of cassette and integron data. BMC bioinformatics. 2009;10:118. doi: 10.1186/1471-2105-10-118.

7. Abu-Ali GS, Ouellette LM, Henderson ST, Lacher DW, Riordan JT, Whittam TS, Manning SD. Increased Adherence and Expression of Virulence Genes in a Lineage of *Escherichia coli* O157:H7 Commonly Associated with Human Infections. PLOS ONE. 2010;5(4):e10167. doi: 10.1371/journal.pone.0010167.

8. Mohawk KL, O'Brien AD. Mouse models of *Escherichia coli* O157:H7 infection and shiga toxin injection. J Biomed Biotechnol. 2011;2011:258185- doi: 10.1155/2011/258185.

9. Zhang CZ, Ren SQ, Chang MX, Chen PX, Ding HZ, Jiang HX. Resistance mechanisms and fitness of *Salmonella* Typhimurium and *Salmonella* Enteritidis mutants evolved under selection with ciprofloxacin *in vitro*. Sci Rep. 2017;7(1):9113. doi: 10.1038/s41598-017-09151-y.

10. Dhanani AS, Block G, Dewar K, Forgetta V, Topp E, Beiko RG, Diarra MS. Genomic Comparison of Non-Typhoidal *Salmonella enterica* Serovars Typhimurium, Enteritidis, Heidelberg, Hadar and Kentucky Isolates from Broiler Chickens. PLoS One. 2015;10(6):e0128773. doi: 10.1371/journal.pone.0128773.

11. Klasberg S, Bitard-Feildel T, Mallet L. Computational Identification of Novel Genes: Current and Future Perspectives. Bioinform Biol Insights. 2016;10:121-31. doi: 10.4137/BBI.S39950.

12. van Schaik W. The human gut resistome. Philos Trans R Soc Lond B Biol Sci. 2015;370(1670):20140087-. doi: 10.1098/rstb.2014.0087.

13. Bengtsson-Palme J, Angelin M, Huss M, Kjellqvist S, Kristiansson E, Palmgren H, Larsson DGJ, Johansson A. The Human Gut Microbiome as a Transporter of Antibiotic Resistance Genes between Continents. Antimicrob Agents Chemother. 2015;59(10):6551.

14. Ryu S, Kim BI, Lim J-S, Tan CS, Chun BC. One Health Perspectives on Emerging Public Health Threats. J Prev Med Public Health. 2017;50(6):411-4. doi: 10.3961/jpmph.17.097.

15. Centers for Disease Control and Prevention. One Health Basics. 2017.