MOLECULAR EPIDEMIOLOGY, PANGENOMIC DIVERSITY, AND COMPARATIVE GENOMICS OF *CAMPYLOBACTER JEJUNI*

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ABSTRACT

MOLECULAR EPIDEMIOLOGY, PANGENOMIC DIVERSITY, AND COMPARATIVE GENOMICS OF *CAMPYLOBACTER JEJUNI*

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Campylobacter jejuni, the leading cause of bacterial gastroenteritis in the United States, is often resistant to commonly used antibiotics and has been classified as a serious threat to public health. Through this work, we sought to evaluate infection trends, quantify resistance frequencies, identify epidemiological factors associated with infection, and use whole-genome sequencing (WGS) as well as comparative phylogenomic and pangenomic approaches to understand circulating *C. jejuni* populations in Michigan. *C. jejuni* isolates (n=214) were collected from patients via an active surveillance system at four metropolitan hospitals in Michigan between 2011 and 2014. Among the 214 *C. jejuni* isolates, 135 (63.1%) were resistant to at least one antibiotic. Resistance was observed for all nine antibiotics tested yielding 11 distinct resistance phenotypes. Tetracycline resistance predominated (n=120; 56.1%) followed by resistance to ciprofloxacin (n= 49; 22.9%), which increased from 15.6% in 2011 to 25.0% in 2014. Notably, patients with ciprofloxacin resistant infections were more likely to report traveling in the past month (Odds Ratio (OR): 3.0; 95% confidence interval (CI): 1.37, 6.68) and international travel (OR: 9.8; 95% CI: 3.69, 26.09).

To further characterize these strains, we used WGS to examine the pangenome and investigate the genomic epidemiology of the *C. jejuni* strains recovered from Michigan patients. Among the 214 strains evaluated, 83 unique multilocus sequence types (STs) were identified that were designated as belonging to 19 previously defined clonal

complexes (CCs). Core-gene phylogenetic reconstruction based on 615 genes identified three clades, with Clade I comprising six subclades (IA-IF) and predominating (83.2%) among the strains.

Because specific cattle-associated STs, such as ST-982, predominated among strains from Michigan patients, we also examined a collection of 72 *C. jejuni* strains from cattle recovered during an overlapping time period by WGS. Several phylogenetic analyses demonstrated that most cattle strains clustered separately within the phylogeny, but a subset clustered together with human strains. Hence, we used high quality single nucleotide polymorphism (hqSNP) profiling to more comprehensively examine those cattle and human strains that clustered together to evaluate the likelihood of interspecies transmission. Notably, this method distinguished highly related strains and identified clusters comprising strains from both humans and cattle. For instance, 88 SNPs separated one cattle and one human strain that were previously classified as ST-8, while the human and cattle derived ST-982 strains differed by >200 SNP differences. These findings demonstrate that highly similar strains were circulating among Michigan patients and cattle during the same time period and highlight the potential for interspecies transmission and diversification within each host.

In all, the data presented illustrate that WGS and pangenomic analyses are important tools for enhancing our understanding of the distribution, dissemination, and evolution of specific pathogen populations. Combined with more traditional phenotypic and genotypic approaches, these tools can guide the development of public health prevention and mitigation strategies for *C. jejuni* and other foodborne pathogens.

To my madrinha and padrinho, João and Madalena Rego, who taught me
“juízo na cabeça”

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KEY TO ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
ARG	Antimicrobial resistance genes
AST	Antimicrobial susceptibility testing
BLAST	Basic local alignment search tool
CAT	Chloramphenicol acetyltransferase
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
cgMLST	Core genome multilocus sequence typing
cgSNP	Core genome single nucleotide polymorphism
CIDT	Culture independent test
CI	Confidence Interval
CLSI	Clinical Laboratory Standards Institute
CMH	Cochran-Mantel-Haenszel
Contigs	Contiguous sequences
ECOFF	Epidemiological cut-off values
EUCAST	European committee on antimicrobial susceptibility testing
FDA	Food and Drug Administration
FoodNet	Foodborne Diseases Active Surveillance Network

FQ	Fluoroquinolone
HGT	Horizontal gene transfer
HIV	Human Immunodeficiency virus
hqSNP	High quality single nucleotide polymorphism
LOS	Lipid oligosaccharide
MDHSS	Michigan department of health and human services
MDR	Multidrug resistance
MDRGI	Multidrug resistance genomic island
MDSS	Michigan disease surveillance system
MIC	Minimum inhibitory concentration
ML	Maximum likelihood
MLK	Macrolide, lincosamides and ketolides
MLST	Multilocus sequence type
NARMS	National Antimicrobial Resistance Monitoring System
NCBI	National Center for Biotechnology Information
OR	Odds ratio
PAM	Partition around medoids
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
PubMLST	Public databases for molecular typing and microbial genome diversity
PulseNET	The National Subtyping Network for Foodborne Disease Surveillance
RND	Resistance-nodulation-division
SAS	Statistical analysis software

ST	Sequence type
SNPs	Single nucleotide polymorphism
SNV	Single nucleotide variant
TSA	Tryptone soy agar
USDA	United States Department of Agriculture
US	United States
WGS	Whole genome sequencing
wgMLST	Whole genome multilocus sequence typing

CHAPTER 1: LITERATURE REVIEW: EPIDEMIOLOGY, ECOLOGY, EVOLUTION, AND GENOMIC DIVERSITY OF ANTIBIOTIC RESISTANT *CAMPYLOBACTER JEJUNI*

Campylobacter spp. are the leading cause of bacterial gastroenteritis in the world and represent the most common cause of bacterial foodborne illness in the United States (US) (1, 2). The gram-negative pathogen has been estimated to cause 1.5 million infections in the US (3). Further, the Centers for Disease Control and Prevention (CDC) report that 13,000 hospitalizations and 120 deaths occur in the United States annually due to this enteric pathogen (2, 4). The active CDC surveillance system, the Foodborne Disease Surveillance Network (FoodNet), estimated that 14 cases of *Campylobacter* gastroenteritis are diagnosed per 100,000 individuals annually. Indeed, *Campylobacter* comprised 34% of all laboratory-confirmed cases identified within FoodNet in 2005 (3, 5, 6). In 2018, the FoodNet reported an incidence of 19.6 cases of campylobacteriosis per 100,000 individuals, which had increased from 12.0 cases per 100,000 in 2015-2017 (7). Campylobacteriosis also results in a significant economic burden with associated costs estimated to be $1.7 billion annually in the US (8).

The genus *Campylobacter* belongs to the family of *Campylobacterales* (9), which represent a large and diverse taxonomic group comprising morphologically distinct and nutritionally fastidious organisms. This genus of bacteria includes organisms that grow under strict anaerobic or microaerobic conditions (10). Several of the first reports of *Campylobacter* were described as “small vibrios” associated with a disease called “cholera infantum” or from the aborted fetuses of sheep and cows (11, 12). In 1938, *Campylobacter* was first isolated from a human infection, and the pathogen at the time was termed “*Vibrio jejuni*” (13). This isolation represented the first recorded outbreak of human disease, in which the later designated genus *Campylobacter* was implicated as the causative agent. The initial diagnosis of “intestinal flu” was made with symptoms

including nausea, vomiting, abdominal cramps, diarrhea, and fever. The outbreak described 357 different infections in two different US prisons and 13 inmates who died. These illnesses were attributed to "*Vibrio jejuni*" isolated from patients (13). In the years that followed, these previously identified vibrios were included in the genus *Campylobacter* with *C. coli*, *C. jejuni*, *C. fetus* and *C. sputorum* representing the first four species identified (10). The initial description of the bacterium was noted by Theodor Escherich in 1886 followed by its isolation in 1906 and the first human isolation in 1938 (11). After its reclassification into a genus in 1963, there have been 36 species and 14 subspecies identified, named, and published within the genus *Campylobacter* (10–15). Several *Campylobacter* species have been shown to cause disease in humans, however, *C. jejuni and C. coli* cause most of the human infections attributable to *Campylobacter* spp. Infection with species such as *C. fetus*, *C. upsaliensis*, *C. lari*, *C. insulaeingrae*, and *C. hyointestinalis*, have been reported but are less common (10). Given the association with human infection and its ability to survive in food animals, this review will focus on *C. jejuni* with an emphasis on the clinical manifestations, ecology, evolution, and genomic epidemiology. Understanding the mechanisms and implications of antimicrobial resistance in this species will also be discussed.

CLINICAL MANIFESTATIONS OF CAMPYLOBACTERIOSIS

Clinical manifestations caused by *C. jejuni* are like those in other bacterial pathogens that cause gastroenteritis and include fever, abdominal pain, vomiting, weight loss, chills, fatigue, myalgia, malaise, and acute watery or bloody diarrhea (2). The incubation period has been reported to be one to four days after exposure, and the

severity of symptoms varies by dose and strain of the bacterium, although the infectious dose has been described to be as low as 360 colony forming units (CFU) (16–18).

The primary symptoms associated with gastroenteritis are abdominal pain and diarrhea. One third of patients also experience influenza-like symptoms such as malaise, myalgia, headache, fever, and chills (19). Stools are often characterized as loose, watery or diarrhea; often campylobacteriosis is associated with blood in the stool. In a small fraction of patients, a colonic disease mimicking acute ulcerative colitis can be apparent, indicating extended disease into the colon and rectum. Most cases present with hemocytes, or microscopic blood, and polymorphonuclear leukocytes, or white blood cells in the stool, which are signs of inflammatory and invasive diarrheal disease (19). Studies have shown that individuals can continue to excrete *Campylobacter* spp., which will be referred to as *Campylobacter* throughout this work, for weeks following an infection with some studies showing continuous bacterial shedding for up to 69 days (19, 20).

Post-infectious immune sequalae such as Guillain-Barré Syndrome, Miller-Fisher syndrome, and reactive arthritis, have been linked to *Campylobacter* gastroenteritis. Other post-infectious gastrointestinal syndromes including inflammatory bowel disease, esophageal and periodontal disease, celiac disease, and colon cancer, have also been associated with *C. jejuni* infections. Additionally, extra-intestinal *C. jejuni* infections have been reported, which can contribute to brain abscesses, meningitis, and bacteremia, all of which may result in systemic infections (10, 21).

EPIDEMIOLOGY OF *CAMPYLOBACTER* IN THE UNITED STATES

Epidemiologic evidence suggests an increase in the global incidence of campylobacteriosis, particularly in industrialized nations such as North America, Europe,

and Australia (2). Data from other areas including Africa, Asia and the Middle East, however, suggest that campylobacteriosis is endemic in these parts of the world (2). In the US, fluctuations in the incidence of disease have been observed. Data from a ten-year period between 1998 and 2008 estimated that 845,024 disease cases were attributed to *Campylobacter* infections, which resulted in 8,463 hospitalizations and 76 deaths (22). FoodNet has reported an increase in *Campylobacter* incidence of 19.6 cases per 100,000 in 2018 as compared to 12.0 cases per 100,000 in 2015-2017 (7). This increase contrasts with the decreasing incidence observed for other bacterial pathogens such as *Listeria*, *Salmonella*, *Shigella*, Shiga toxin-producing *Escherichia coli* (O157), and *Yersinia* during the same time-period (23).

Among 8270 travel-associated cases gastroenteritis reported between 2004 and 2009, *Campylobacter* was the leading cause accounting for over 40% of these cases, and the second leading cause of non-travel associated gastroenteritis, accounting for over 26% of the cases (2, 24). In the US during 1996 – 2005, 70% of the 7442 gastrointestinal infections acquired during travel overseas were attributed to *Campylobacter* (25). The risk for acquiring *Campylobacter*, however, differs by location, as an increasing risk of infection was linked to travel from a non-endemic country to endemic countries with a high incidence of *Campylobacter*. A Dutch study conducted in 2013, for instance, identified the travel-associated risk of acquiring *Campylobacter* to be highest when traveling to countries in South Asia, Southeast Asia, and China as well as sub-Saharan Africa as compared to traveling to western European countries (26).

Through monitoring of FoodNet data, a distinct seasonality in *Campylobacter* infections has been observed in the US. Most cases were reported in the summer months

(June through August) with case numbers peaking in July. In fact, from 1997 – 2012, 38% of cases were reported in the summer months (27). Seasonal incidence variations have been attributed to ecological phenomena such as higher levels of poultry contamination in the warmer months and eating patterns in the summer like grilling, barbecuing, and outdoor dining (27, 28). Climate, temperature, increased shedding from other animal reservoirs, and/or seasonal-specific behaviors have been suggested to contribute to seasonality in *C. jejuni* infections in other geographic locations as well (29–31).

Age specific variations and risk factors for campylobacteriosis have also been described. From 1997-2012, the incidence of *Campylobacter* was highest for infants (0-6 months) and risk factors for this age group included drinking well water, riding in a shopping cart next to poultry or meat, visiting or living on a farm, having a pet, and eating raw fruits and vegetables (27, 32, 33). Males were reported to have a higher incidence of *Campylobacter* than females in the US and several other countries as well (34). As infection rates were identified by sex in different nations and age groups, it was suggested that susceptibility to *Campylobacter* may be influenced by sex (34). However, gender-associated behavioral differences in hygiene practices involving food consumption, preparation, and handling have also been implicated in this risk association (27, 28). Other risk factors for *Campylobacter* infections include international travel, immune status, consumption of contaminated foods such as undercooked chicken and unpasteurized milk products and contact with farm animals and livestock (35).

Campylobacter has a diverse host range with multiple reservoirs including avian species such as wild birds, poultry, and livestock (2). Therefore, contamination of food producing animals and direct contact with these reservoirs along with environmental

spillover such as the contamination of water sources, are important for *Campylobacter* acquisition (36). The most common source of *C. jejuni* and *C. coli* in the US is contaminated poultry as they can asymptomatically colonize poultry in the cecum at densities as high as 10^{10} CFU per gram of caecal contents (37). Dissemination and transmission within poultry flocks occurs rapidly, where one contaminated bird can result in the contamination of all chicks in a flock within 3 days (38). Further, rapid colonization of chicks was shown to occur following exposure to contaminated water, feed and other chickens ingesting feces (37). *Campylobacter* also resides in cattle as *C. jejuni* has been variably isolated from the caecum as well as the small and large intestines (39). While *C. jejuni* is often isolated from cattle and sheep, *C. coli* is primarily isolated from pork and swine.

Raw and unpasteurized milk products can become contaminated with *Campylobacter* from dairy cattle via fecal contamination (2), which can occur via direct transmission of *Campylobacter* among cows with sub-clinical mastitis (40). Identification of these sources, however, can be challenging as only a subset of dairy cows shed the pathogen at any given time (41, 42). Although *Campylobacter* has been shown to survive several weeks at 4^{o} C, it cannot grow in milk alone (42). Raw milk is a source for *Campylobacter* outbreaks and recent studies have shown that cattle and milk associated outbreaks represent a larger share of source attribution of *Campylobacter* in the US (22, 43, 44).

Several studies have also identified water sources as important reservoirs for *Campylobacter*. In fact, *C. jejuni*, has been observed to survive in water for several weeks or months, and drinking contaminated water is among the most common causes of

Campylobacter outbreaks globally (2). An outbreak involving 116 confirmed *C. jejuni* cases, for instance, was in Walkerton, Canada where heavy rainfall flushed cattle manure from an adjacent farm into municipal water supplies (45). Several outbreaks of contaminated municipal water sources have been described in the US and world (46–54) and thus, contact with fecally contaminated water is an important risk factor linked to the environmental acquisition of both sporadic and outbreak associated *Campylobacter* infections.

Immunodeficiency, and more specifically, HIV status is an important risk factor for campylobacteriosis. Prior studies have shown that HIV-positive patients more frequently present with *Campylobacter* and have a higher incidence of *Campylobacter* related illnesses than the individuals without HIV (55, 56). Patients with defects in either the innate or adaptive immune responses are at risk for complications like bacteremia and are more prone to recurrent infections and chronic *Campylobacter* carriage. This chronic colonization state has been reported to last up to several months, and in some reports, patients were recurrently infected or carrying *Campylobacter* for up to six years (57, 58). Indeed, *C. jejuni* has been implicated in a long-term (ten year) outbreak in a population of men who had sex with men in Quebec, CA (59). In a recent report utilizing whole-genome sequencing (WGS), a 15-year symptomatic infection was described in a patient with combined variable immunodeficiency (60).

Immune status could explain some differences in *Campylobacter* incidence across populations and geographic regions (2). Population-level immunity refers to a host response at the population level, which is termed herd immunity. Herd immunity can contribute to how infectious diseases may transmit and impact unprotected individuals

within a particular community or population (61). In nations with endemic levels of *Campylobacter*, infections predominate in children and symptomatic disease is often restricted to this age group (62). These observations, amongst others such as the prevalence of asymptomatic *Campylobacter* colonization in non-endemic nations as well as adaptive and innate immune deficiencies that contribute to long-term carriage of the pathogen, highlights the importance of early exposure on immunity (63–65). In fact, it has been theorized that multiple repeated infections with genotypic and phenotypic diverse *Campylobacter* strains contribute to immune protection for future *Campylobacter* infections (66). Indeed, multiple antigens, the interplay between the innate and adaptive immune response, and the clearance effect of *Campylobacter* specific antibodies have been shown to be important for protection (63, 67–69).

HEALTH DISPARITIES IN FOODBORNE AND OTHER INFECTIOUS DISEASES

Distinct disparities in the incidence and clinical outcomes across populations are not unique to *Campylobacter* infection. In fact, disparities in disease burden are a hallmark of infectious disease epidemiology. Dense crowded locations, lack of institutionalized sanitation systems or access to clean running water, increased exposure to domesticated wildlife, and a poor public health infrastructure for outbreak detection and food safety all contribute to differences in the incidence and endemicity of foodborne infection across nations (70). In the US, poverty and neighborhood resources are critical to public and personal health and thus, infrastructure and individual interactions with institutions can impact the propensity for diseases. Specific populations have been differentially impacted by infectious disease burdens because of the disproportionate

distribution of resources, and the current demographics of neighborhoods that have sustained investment or divestment (71).

Access to healthcare and interlinked co-morbid conditions also play a role in health outcomes in the US. For example, Black and Brown individuals have been unduly impacted by respiratory infectious diseases and experience poorer health outcomes during outbreaks, epidemics, and pandemics, such as the one linked to the severe acute respiratory distress syndrome from Coronavirus 2 (72, 73).

Neighborhood and geographic barriers are intimately related to the acquisition of foodborne disease (74). Although race, ethnicity, and other socially constructed categorizations such as socio-economic status, are not typically collected in foodborne disease surveillance systems, several global studies have shown increased frequencies of gastrointestinal diseases in minority and low-socioeconomic populations (75–78). Increased incidence of specific foodborne disease caused by different pathogens has been attributed to specific food consumption behaviors. For instance, *Yersina entercolitica* causes more infections in African American populations due to chitterling consumption, while *Listeria monocytogenes* has been linked to higher rates in Hispanic populations due to the consumption of Mexican style soft cheeses (79–81). Although individual and cultural behaviors contribute to an increase in consumption of such foods, other disparities are likely important for *Campylobacter* and other prevalent foodborne infections such as Shiga toxin-producing *E. coli*, and *Salmonella*. For instance, critical prevention efforts should focus on the farm to fork interface to reduce disparities as minority populations are disproportionality acquiring their food in “Food Deserts” (82, 83). These deserts refer to areas that have a shortage of larger retailers and more small

independent retailers as well as fast food retailers, however, few studies have assessed differential food safety risks in food deserts (74, 82).

FoodNet data from 1996-1999 shows that the highest incidence of campylobacteriosis was reported for Hispanics at 10.2 – 11.5 cases per 100,000 individuals, while African Americans had the lowest incidence at 2.0 – 3.6 cases per 100,000 individuals (74). Comparatively, those identifying as Caucasian had a collective incidence of 8.9 – 12.2 per 100,000 individuals, though it is not clear whether these older frequencies are consistent with recent data, while newer studies are needed to continue to explore these critical risk factors.

It is also critical to examine differences by environment such as in urban versus rural settings, as these may possess differential risk factors for disease, which was described previously for Shiga toxin-producing *Escherichia coli* and *Salmonella* infections (84). Although differences in incidence across socially constructed categories may be due to cultural differences in food consumption, other factors such as exposure to or direct contact with reservoirs, travel to *Campylobacter* endemic areas, and more broadly, neighborhood and societal divestment in areas that demographically are a majority minority, are also important. In Michigan from 2014-2015, Black and Brown individuals were disproportionately impacted by a *Legionella* outbreak associated with the Flint water crisis (85). Institutional injustices such as structural racism and the legacy of slavery in the US may explain sustained divestment in neighborhoods that disproportionately impact disease rates in Black and Brown communities (86). These institutional barriers and historical perspectives may underscore the mistrust of medical and broader public health infrastructure within impacted communities. Attempts to mitigate health disparities for

these populations should and must be grounded within the community and citizen engagement framework, which will enable communities to be active participants in finding solutions to these disparities (87).

TREATMENT OF *C. JEJUNI* AND MODE OF ANTIBIOTIC ACTION

Although bacterial gastroenteritis caused by *C. jejuni* is typically self-limiting, antibiotic treatment is required for prolonged diarrheal illness, invasive and complicated infections such as bacteremia or extra-intestinal infections (19). Electrolyte imbalance, maintenance of fluids, and hydration are important considerations when treating all cases of gastroenteritis including those attributed to *Campylobacter*. Macrolides and fluoroquinolones are the primary and secondary drugs for treating campylobacteriosis, though the aminoglycosides have been used for complicated and extra-intestinal infections (10, 88). By contrast, the tetracyclines have been discontinued and are no longer recommended for use, and the β-lactams were shown to be ineffective for treating *Campylobacter* infections (10, 88).

The fluoroquinolones (FQs)

The FQs, particularly ciprofloxacin, have broad spectrum activity and thus, these antibiotics are often used empirically for the treatment of enteric disease and unexplained diarrheal illness (89). FQs are chemically modified versions of the antibiotic nalidixic acid, a quinolone. These antibiotics have two bacterial targets, DNA topoisomerase IV and DNA gyrase (type II topoisomerase), which are important for bacterial replication (10, 90–92). FQs are bacteriostatic inducing complex formation of these proteins, preventing DNA replication, and poisoning the bacterial cells resulting in apoptosis or cell death (10).

The macrolides

Macrolides are considered clinically safe and effective and are recommended for the treatment of campylobacteriosis. These bacteriostatic antibiotics halt protein synthesis by binding to the bacterial ribosome or specifically, the 23S rRNA and proteins such as the L4 and L22 (93–96). This class of antibiotics, which includes erythromycin, inhibits the elongation step of protein synthesis at transpeptidation by causing dissociation of the peptidyl-tRNA in the ribosome (97) . The closely related macrolide-like classes of antibiotics including the lincosamides, clindamycin and lincomycin, the ketolides, azithromycin and clarithromycin, the streptogramins, and oxazolidinones, also interrupt protein synthesis by ribosomal binding. Macrolides are large molecular weight (>700) hydrophilic molecules. These antibiotics gain access to the cytoplasmic membrane through the hydrophobic pathway (90). As compared to other gram-negative bacteria that contain lipopolysaccharide (LPS), *Campylobacter* lack hydrophilic moieties and thus, they possess a lipooligosaccharide (LOS) membrane structure, which is more hydrophobic (10, 98–104). This comparative increase in hydrophobicity likely impacts the uptake of hydrophilic molecules such as macrolides, which could explain the susceptibility to macrolides observed among *Campylobacter* (105).

The tetracyclines

Tetracyclines are broad spectrum antibiotics that were previously used in both human and veterinary medicine (106). Antibiotics in this class include chlortetracycline, tetracycline, doxycycline, minocycline, oxytetracycline and the newest antibiotic glycylcycline. Tetracycline antibiotics are protein synthesis inhibitors that gain access to

the ribosome through hydrophobic pathways and porins. Once in the cytoplasm of the bacterial cell, such as that of *Campylobacter*, tetracyclines prevent the attachment of aminoacyl-tRNA to the A site of the ribosome by reversibly binding to the ribosome (107, 108).

Other antibiotic classes

β-lactam antibiotics are a large diverse class of antibiotics that include several sub-families including penicillin, cephalosporins, carbapenems and monobactams. These sub-classes of antibiotics have varying properties as well as differing activity against gram-negative or gram-positive bacteria (109). The diverse properties of β-lactam antibiotics such as pharmacokinetics and resistance to β-lactamase enzymes and stomach acid, are mediated by a variety of diverse chemical sidechains. The β-lactam ring is required for activity as it inactivates the bacterial peptidoglycan penicillin binding proteins, the transpeptidases. Consequently, the structural integrity of the bacterial cell wall gets disrupted, and the cells lyse due to osmotic stress (110–113).

Campylobacter infection can also be treated with more toxic antibiotic classes such as phenicol, chloramphenicol and aminoglycosides, which include gentamicin, kanamycin, amikacin, neomycin, tobramycin, and streptomycin (100). Aminoglycosides require active transport into the bacterial cell through oxygen, and *Campylobacter* remains susceptible despite being microaerophilic (90). Once in the cell the antibiotic reversibly binds the 30S segment of the ribosome disrupting translocation that leads to premature termination of peptide formation, and disruption of proof-reading functions. The

result is dysfunctional protein formation. Phenicol and chloramphenicol are also protein synthesis inhibitors that bind the 50s ribosome and inhibit protein synthesis (90, 114).

ANTIBIOTIC RESISTANCE IN *C. JEJUNI*

Another critical factor contributing to differential health outcomes among patients with infectious diseases caused by bacterial pathogens such as *Campylobacter*, is antibiotic resistance. Bacterial resistance to antibiotics has been described as a global health threat. The CDC has estimated that approximately two million resistant infections occur annually in the US alone, contributing to 23,000 deaths (2, 115, 116). Projections suggest that antimicrobial resistance (AMR) could be a leading cause of death worldwide and account for 10 million deaths by the year 2050. Further, in the US, the CDC has estimated the total cost of AMR to be $55 billion, with $20 billion due to excess healthcare costs and another $35 billion due to losses due to productivity (117).

Importantly, the CDC has classified antibiotic-resistant *Campylobacter* as a serious public health threat, contributing to 500,000 infections per year (118). Antibiotic resistance emerged in *C. jejuni* and other bacterial pathogens through natural evolutionary processes. Within environments exposed to antibiotics, susceptible bacteria are inhibited and killed, thus allowing for cells that are intrinsically resistant or that have acquired resistance to be selected for and replicate (119). Antibiotic use promotes the emergence of resistance in both the target bacterial pathogen and the normal (commensal) members of a given bacterial community (120–123).

Resistant pathogens may contaminate food at the time of slaughter and be transmitted to humans resulting in drug-tolerant, resistant, and multi-drug resistant infections, which are more difficult to treat (124). Selection occurs in environments

occupied by *C. jejuni* and other bacterial pathogens when antibiotics are present as the susceptible populations cannot survive. Therefore, risk factors for resistant foodborne infections have been shown to mimic those of susceptible infections and direct contact, contamination of drinking water, and consumption of contaminated food products are most important (125). Resistance has become a worldwide problem and classes of antibiotics used in human and food-producing animals can overlap, thereby increasing the likelihood of selecting for resistant pathogens in both human and veterinary medicine (84). Overprescribing in primary care and hospital settings is of particular concern as 90% of all antibiotic prescriptions in human health are prescribed by general or primary care practitioners. Respiratory infections are the primary reason for prescribing antibiotics despite most respiratory illnesses having viral etiologies. Although selection of resistant pathogens can occur in humans during antibiotic treatment, it more commonly occurs in animal reservoirs (126). Overuse of antibiotics in veterinary and human medicine as well as agriculture has resulted in the emergence and dissemination of resistant foodborne pathogens in multiple reservoirs and environments (10).

Using a One Health framework investigators have studied the compilation of antimicrobial resistance genes (ARGs) that are distributed within microbial communities. Studies have identified the compilation of ARGs, the resistome, in various ecological niches including humans, and livestock such as cattle, poultry and swine. ARGs have also been identified in environmental soil isolates and multiple human pathogens (84, 127). These genes have also been shown to be co-localized with mobile genetic elements that can be readily transferred across environments (117, 122). Consequently, pathogens such as *C. jejuni* are important reservoirs for ARGs.

Expansion of resistant lineages that carry resistance determinants as well as horizontal gene transfer (HGT) play critical roles in the dissemination of resistance determinants that contribute to increasing pathogen resistance frequencies. The excessive use and misuse of antibiotics contribute to the selection of antibiotic resistant mutations and particular lineages that carry ARGs, or antibiotic resistance determinants (10, 90, 114). In *Campylobacter*, the bacterial mechanisms that mediate antibiotic resistance to a variety of antibiotics include target modification, target protection, reduced permeability, efflux of the antibiotic, and modification or inactivation of the antibiotic (4, 128, 129). These mechanisms, however, vary by antibiotic, species, and strain.

To detect resistant infections, antimicrobial susceptibility testing has been standardized using the guidance and standards established by the Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (128). In 1996, the US established the National Antimicrobial Resistance Monitoring System (NARMS), a surveillance system comprising a collaborative network of local, regional, state, territorial and national public health agencies (10). The primary goals of NARMS are to investigate and monitor national trends of antibiotic resistance utilizing established clinical laboratory standards. NARMS began using epidemiological cut-off (ECOFF) standards and breakpoints established by EUCAST for detecting *Campylobacter* resistance in 2012 (130, 131). This standardized approach was adopted to enable global surveillance of *Campylobacter* resistance and comparisons across populations and geographic locations.

Mechanisms of fluroquinolone (FQ) resistance

Modification of the quinolone or FQ target site on the gyrase A (GyrA), which is encoded by *gyrA*, confers resistance to FQs. Mutations in *gyrA* within the quinolone-resistance determining region (QRDR) or the DNA binding domain of the protein, lead to functional disruptions of GyrA that include reduced DNA supercoiling (132). Several non-synonymous single nucleotide polymorphisms (SNPs) have been identified to confer resistance to FQs including those causing changes in residues Ala-70, Asp-85, Thr-86, Asp-90, and Pro-104, which are in the QRDR of GyrA (133–135). These mutations have been identified in multiple combinations, although Thr-86-Ile (C-257-T) mutations are most common and result in higher minimum inhibitory concentrations (MICs) as compared to the other five mutations identified. Each mutation was shown to confer cross-resistance to quinolones and nalidixic acid, though the Thr-86-Ile mutation confers resistance to eight FQs including ciprofloxacin, levofloxacin, danofloxacin, difloxacin, enrofloxacin, gatifloxacin, marbofloxacin, and orbifloxacin (136). Recombinant enzymes with mutations in *gyrA* that mediate FQ resistance in *C. jejuni* have also been shown to have a reduced ability to supercoil DNA, which mediates global transcriptional changes in the bacterium (137–139). Unlike other gram-negative bacteria with GyrA, gyrase B (GyrB), and topoisomerase IV enzymes (ParC/ParE), *C. jejuni* possesses topoisomerase type II enzymes only; thus, a single mutation in *gyrA* is sufficient for clinical resistance to FQs (140). Although *gyrB* mutations have been reported, they are not typically implicated in FQ resistance in *Campylobacter* (141).

The “one-hit” nature of resistance to this antibiotic leads to rapid selection of FQ resistant mutants (140). FQ resistant *C. jejuni* have been shown to persist within bacterial

populations even in the absence of FQs (selective pressure) since the *gyrA* mutations do not impact survival (10, 141). In the poultry reservoir, for instance, FQ resistant *C. jejuni* are likely sustained in the population because of this rapid selection and their apparent fitness benefit as compared to antibiotic susceptible *C. jejuni* (133). This fitness benefit is mediated by a mechanistic change in the bacterium that reduces DNA supercoiling, which has been linked to transcriptional changes (10, 92, 142). Indeed, Thr-86-Ile mutants were observed to have enhanced biofilm formation, motility, stress tolerance, and invasion of human epithelial cells (143–145). Thus, these data could partly explain the persistence of the mutation as well as epidemiological data showing poorer health outcomes among patients with FQ resistant *C. jejuni* infections as compared to FQ susceptible *C. jejuni* infections (146).

Efflux of FQ outside of the cell is another important mechanism for FQ resistance in *Campylobacter*, which typically have two efflux systems, CmeABC and CmeDEF (147). These efflux systems are like other resistance-nodulation-division (RND) systems in gram-negative bacteria as they contribute to intrinsic resistance to multiple antibiotics, general homeostasis, and stress tolerance (143). CmeDEF plays a modest role in intrinsic FQ resistance (143), whereas CmeABC works synergistically with point mutations within the QRDR to confer high levels of FQ resistance (148). Unlike other gram-negative species, *Campylobacter* do not need to overexpress RND efflux systems to confer resistance to FQs (149). However, studies have shown that mutations within the CmeR repressor binding site impact transcriptional activity of the CmeABC efflux system resulting in higher MICs to multiple antibiotic classes (90, 140). Disruption of these systems results in the accumulation of antibiotics within the bacterial cell, thereby

resulting in antibiotic susceptibility (10, 140). These data therefore suggest that CmeABC contributes to multidrug (MDR) resistance and is an important resistance determinant for all classes of antibiotics including the FQs (150). *cme* overexpressing mutants have not been reported to be globally distributed (10, 140) and thus, the propagation and maintenance of *cmeABC* overexpression in the absence of strong selection pressures requires further study. Although there could be fitness costs for overexpressing RND systems, reports in other gram-negative bacteria such as *Pseudomonas aeruginosa*, show compensatory mechanisms that result in “metabolic rewiring” (151). This rewiring in RND overexpressing gram-negative bacteria might explain why there is not a dramatic decrease in fitness among antibiotic resistant versus susceptible populations in the absence of antibiotic selection (151–159).

Mechanisms of resistance to macrolide and macrolide-like antibiotics

In *Campylobacter,* mechanisms of resistance to macrolides, lincosamides and ketolides (MLK) antibiotics include target mutation and modification as well as efflux and altered membrane permeability. Ribosomal mutations that confer resistance to MLK-antibiotics are found in 23S *rRNA* and other genes encoding ribosomal proteins (94). Mutations in nucleotide positions 2074 and 2075 that include A2075G and A2074T, are most common and result in the highest MICs to MLK-antibiotics. In combination with mutations in genes encoding the L4 and L22 ribosomal subunit proteins, these mutations confer higher MICs to MLK-antibiotics but are differentially effective at conferring resistance to all the MLK-antibiotics (93, 160, 161). In 2014, reports from China indicate that 2-5% of macrolide resistant *Campylobacter* isolates containing the A2075G/T

mutation are susceptible to the lincosamides and clindamycin (162, 163). Furthermore, *C. coli* and *C. jejuni* carry up to three copies of the 23S rRNA gene and therefore, multiple "hits" or mutations are required for resistance. Indeed, a dosage effect has been suggested for these mutations as some reports have identified lower MICs to macrolides with only one or two mutations (10, 164–166).

HGT has also played an important role in the dissemination and propagation of MLK resistance in *Campylobacter*. It was suggested that the transfer of genes containing the multidrug resistant island (MDRGI) likely occurred from gram-positive bacterial populations (167–169). *ermB*, which encodes for a ribosomal methylase, is found on the MDRGI and confers inducible resistance to MLK antibiotics. High MICs are achieved through *ermB* methylation of an adenine in the 23S RNA component of the 50s subunit of the ribosome. *ermB* expression is modulated via antibiotic binding to the ribosomal protein (170). Protective modification of the ribosomes through *ermB* methylation was described in *Campylobacter* but seems to be geographically restricted since *Campylobacter* isolates containing this gene have yet to be identified in the US (90). The RND systems in *Campylobacter* also play a critical role in resistance to macrolides. CmeABC, for instance, has been implicated in low levels of intrinsic resistance that is linked to synergistic 23S rRNA mutations, particularly in *C. coli* (171, 172). Alterations of membrane permeability also play an important role in resistance to macrolides (173, 174).

Tetracycline resistance

Known tetracycline resistance mechanisms in *Campylobacter* include ribosomal protection and efflux of the antibiotic out of the bacterial cell. Resistance in *Campylobacter*

is primarily driven by the presence of *tet(*O) found on conjugative plasmids, integration within the chromosome has been described (10, 90). The Tet(O) soluble protein actively displaces tetracycline from the ribosomal binding site. The N-terminal region of Tet(O) has GTPase activity and sequence similarity with elongation factors such as EF-G (10). Hence, Tet(O) promotes displacement of tetracycline by inducing a confirmational change in the ribosome that releases tetracycline from the ribosomal binding site. Efflux also plays an important synergistic role in mediating resistance to tetracycline. Although Tet(O) sufficiently results in high MICs to tetracyclines, disruption of *Campylobacter* efflux systems contributed to a 4-8-fold increase in susceptibility to tetracycline (10, 100, 105, 175, 176).

Tetracycline plasmids such as pTet and pCC31, are also common in *C. jejuni* and *C. coli* isolates, respectively (177). One report suggests that carriage of these plasmids has a neutral impact on *Campylobacter* fitness; yet, other plasmid-associated genes could confer similar compensatory fitness benefits (178). While genes encoding type-IV secretion systems have also been found on pTet plasmids, these were suggested to be important for conjugation (176, 179, 180). Furthermore, *tet*(O) genes have been found on pVIR plasmids, which are important mediators of virulence in *Campylobacter* (181–183).

Additional mechanisms of antibiotic resistance

Resistance to β-lactams is often mediated through the enzymatic activity of β-lactamases that cleave the β-lactam ring to inactivate the drug (10, 90). Efflux and permeability also play a role in β-lactam resistance as was shown for many other antibiotic classes (100, 184). *Campylobacter* is intrinsically resistant to penicillin and narrow

spectrum cephalosporins, while resistance to amoxicillin, ampicillin and ticarcillin are mediated by penicillinases that are readily expressed (110, 112, 113, 185). National data on β-lactam resistance in *Campylobacter* is not available as these antibiotics are not regularly tested for susceptibility via NARMS using standard microbroth dilution methods (185). Nonetheless, early reports showed moderate to high MICs to several classes of β-lactams in *Campylobacter* as well as high rates of carriage of β-lactamase genes. To overcome resistance mediated by β-lactamases, inhibitors such as the penicillinases, can recover susceptibility to these antibiotics in *Campylobacter* (186). Class D β-lactamase OXA-61 has also been identified and confers resistance to ampicillin, amoxicillin-clavulanate, piperacillin, and carbenicillin (187). Also, there have been reports of genes encoding other metallo-β-lactamases in *Campylobacter*, the expression and effect of these genes on resistance is not yet clear (90, 188, 189).

Aminoglycoside modifying enzymes are another important way that resistance is mediated in *Campylobacter*. For example, the 3'-aminoglycoside phosphotransferase encoded by *aphA-3* is a common cause of aminoglycoside resistance in *Campylobacter* (190, 191). These genes are typically found with other aminoglycoside resistance genes such as *aadE* encoding a 6'-adenylyl transferase conferring resistance to streptomycin, and *sat*, which encodes an acetyl transferase for resistance to streptothricin (192). Each of these genes are often found on plasmids along with *tet*(O). Additional studies have also found transposon sequences adjacent to these genes, which is suggestive of inter-genus HGT (192, 193). Because *aphA-7* has a similar GC content as the *Campylobacter* chromosome, it was hypothesized that this gene is intrinsic to *Campylobacter* (194). Comparatively, the GC content of *aphA*-1 and *aphA*-3 are higher than that of the

Campylobacter chromosome, which is more indicative of gene acquisition. Lastly, mutations in the ribosomal protein S12 encoded by *rpsL*, which contributes to streptomycin resistance, were recently detected in *C. jejuni* (195).

For resistance to phenicol and chloramphenicol, the primary mechanism is compound modification by *cat* encoding a chloramphenicol acetyltransferase (CAT) (196–200). In addition, *cfr*(C), which encodes for a rRNA methyltransferase, was identified in *C. coli* to be important for resistance to phenicol, lincosamides, oxazolidinones, plueormutilins and streptogramin A. This gene is found on plasmids and it is thought to have originated from *Staphylococcus sciuri* (195). Finally, efflux systems have been shown to be important for phenicol resistance as *Campylobacter* isolates from China had a point mutation in the inter-gene binding domain of the CmeR repressor protein. This mutation contributed to increased expression of the CmeABC efflux system resulting in high MICs to phenicol (23, 115).

EMERGENCE OF ANTIBIOTIC RESISTANCE IN *C. JEJUNI*

The emergence and maintenance of fluroquinolone resistance

Since the introduction of FQs such as ciprofloxacin in the 1980s, this antibiotic class was used widely to treat gastroenteritis caused by *C. jejuni* (153); however, FQ resistant *C. jejuni* quickly emerged after FQs were approved for use in humans and food production (157, 158). The FQ enrofloxacin, which was not typically used to treat human infections, was introduced in 1987 to animal industrial settings to treat chicken infections. Importantly, the study by Endtz et al. (153) in the Netherlands identified a temporal and spatial relationship between the introduction of enrofloxacin, where no FQ resistant *Campylobacter* isolates were identified from 1982-1987, and the recovery of FQ resistant

isolates from both humans and chickens. In the subsequent years in Netherlands (1987-1989), the frequency of FQ resistant *Campylobacter* increased to 11% among human isolates and 14% among chicken isolates (155, 156, 201). Similarly, remarkable evidence from Spain showed that FQ resistance frequencies from pediatric patients increased from 0-2% before 1989 to 88% in 1996 (151, 152, 158). In Canada frequencies of FQ resistance was reported to have increased from 0% before 1989 to 13% in 1995 (152), while reports of FQ resistant *Campylobacter* infections quickly followed in the US (202). Indeed, researchers from the Minnesota Department of Health reported an increasing frequency of FQ resistant *Campylobacter* infections from 1992-1998 (203). A significant increase in total FQ resistant *Campylobacter* infections from 1.3% in 1992 to 10.2% in 1998 was observed among the 4,953 *Campylobacter* isolates submitted. Moreover, a significant increase in domestically acquired FQ resistant *Campylobacter* isolates infections was observed with frequencies of 0.8% in 1996 and 3.0% in 1998 (204).

To determine the source of these resistant *Campylobacter* infections in humans, one study compared the isolates recovered from clinical infections to those from local retail poultry products (205). The researchers identified identical polymerase chain reaction (PCR)-based restriction-fragment length polymorphism (RFLP) profiles among the *Campylobacter* isolates from both sources, thereby providing evidence of direct transmission of highly similar strains (206). Thus, the research proposed an animal use hypothesis, where FQ use in poultry promoted the emergence and selection of antibiotic resistant *Campylobacter* populations that could contaminate food products post-harvest.

Following these early reports, additional studies supporting the animal use hypothesis were conducted. For instance, one study in the US demonstrated increasing

frequencies of FQ resistant *Campylobacter* infections in humans from 1997-2001, while another found that 10% of 6,138 retail chickens samples collected in the US were contaminated with FQ resistant *Campylobacter* from 2002 and 2007 (207, 208). Based on these and additional studies (92, 130, 152, 153, 157, 201, 209, 210), the Food and Drug Administration (FDA) estimated that more than 10,000 FQ resistant *Campylobacter* infections were the result of FQ use in the poultry industry in the US (211). Hence, enrofloxacin was prohibited for use in poultry in 2005, which was the first time that an antimicrobial was banned because of evidence indicating its importance in the emergence of an antibiotic resistant pathogen (212). More recent data from 1997-2012 suggests a continued increasing trend of FQ resistance among *Campylobacter* infections in the US even though enrofloxacin and other FQs are no longer used during poultry production (211). Moreover, data reported in 2018 show that 28.8% of the 1,226 *C. jejuni* isolates from human infections examined at the nine participating NARMS sites were resistant to FQs (4).

There are several reasons for the continued occurrence of FQ resistant *Campylobacte*r infections in the US. The persistence of FQ resistant *Campylobacter* has been attributed in part to co-selection of *gyrA* mutants, which have increased in frequency and have replaced the susceptible *gyrA* allele in poultry flocks (212). Indeed, studies have identified carriage of similar genotypes of FQ resistant *C. jejuni* in several cycles of broiler flocks. The authors noted that poor sanitation in these environments, with insects and domesticated animals serving as possible intermediate hosts or reservoirs, could have been important in the transfer of FQ resistant *C. jejuni* between flocks in these production facilities (202, 206, 212–214).

In addition to poultry and cattle, unpasteurized milk products from dairy cattle have become increasingly important for the dissemination of resistant *Campylobacter* infections (215). Recent US data from 2017, however, also demonstrated increasing FQ resistance frequencies among *Campylobacter* recovered from beef cattle (10, 187, 205, 213, 215–217). Thus, prior use of FQs in food producing animals other than poultry may also be important for the maintenance of FQ resistance in *Campylobacter* within the agricultural setting. FQ resistance in *Campylobacter* has been well studied and policies mitigating use of FQ in poultry are viewed as a successful example of how policy can mitigate resistance frequencies in pathogenic bacteria. Reductions in FQ resistance have also been observed for other enteric pathogens including diarrheagenic *E. coli* and *Salmonella* spp. since the FQ ban in poultry (218), highlighting the broader impact of mitigation policies. The persistence of FQ resistance in the US is multifactorial and can be attributed in part to the biological fitness benefits of *gyrA* mutations as compared to susceptible *Campylobacter*. Additionally, persistence of FQ resistant *Campylobacter* in poultry flocks and increasing FQ resistance within other pathogen reservoirs such as cattle and various environmental niches, have contributed to persistence.

It is also important to consider the fraction of FQ resistant isolates that are not acquired domestically and are travel associated (100, 105, 175). International travel is a risk factor for FQ resistant *C. jejuni* infections and 13% of all FQ resistant infections reported in the US are travel associated (213). Indeed, there is increased risk of acquiring FQ resistant infections in *Campylobacter* endemic nations that do not have regulations on FQ use in food producing animals and within the environment (2, 219). International

acquisition of resistant *Campylobacter* has the potential to increase the diversity and distribution of resistance determinants in a given area.

The emergence of macrolide resistance

Macrolide resistance rates are lower than FQ resistance rates in *Campylobacter*, however, resistance to MLK-antibiotics is geographically restricted. In the US, Canada, Europe, and China where resistance monitoring is regularly conducted, frequencies of resistance are typically less than 10% of human isolates tested within a given timeframe (100). Some regions of Europe have reported higher frequencies of macrolide resistance as one study observed resistance to MLK-antibiotics in up to 40% of *C. jejuni* isolated from broiler chickens (2). Consequently, travel has been reported to be a risk-factor for macrolide resistant *C. jejuni* infections (10, 90, 140). Data from the US from 2008, however, reported that only ~4.6% of travel associated *C. jejuni* infections were resistant to macrolides (220–224). Additional, more widespread studies are therefore needed.

C. coli typically has higher antibiotic resistance rates than *C. jejuni* (225) as isolates recovered from swine showed macrolide resistance frequencies ranging from 14% to 53%. NARMS also reported that frequencies of resistant *C*. *coli* isolated from chickens and humans increased from 4.5% to 13% during 2011- 2015, while resistance in *C jejuni* remains below 10% among human infections during the same time-period (159). Links between the selective pressure of antibiotic use in animal husbandry and resistance have been made, however, frequencies of resistance to MLK-antibiotics have not risen as dramatically as FQ resistance rates. Studies have linked this discrepancy to geographic restriction of circulating lineages possessing the *ermB* resistant determinant, less

antibiotic use, the need for multiple mutations, and the comparative fitness costs of these resistance mutations (90, 159). Unlike FQ resistant strains, macrolide resistant *C. jejuni* strains are unable to outcompete their wild-type counterpart in competition experiments in the chicken gut (223). In China, where use of macrolides in animal husbandry is common, 73% to more than 98% of *C. coli* strains isolated from chickens are resistant to macrolides (94, 130, 159). Hence, selective pressure, HGT, and genetic background all play important roles in the maintenance and dissemination of MLK-antibiotic resistance in *Campylobacter*.

The emergence of tetracycline resistance

Tetracycline resistance is widespread in *Campylobacter* and other gram-negative and gram-positive organisms, which is likely due to widespread tetracycline use in human and veterinary medicine as well as industry since its discovery in the 1940s (10, 226). Several reports have described widespread tetracycline resistance in both human and animal derived isolates (226). Resistance to tetracyclines has been reported for 50-100% of *Campylobacter* depending on the isolation source (140), yet tetracyclines are no longer effective for the treatment of *Campylobacter* infections in humans (90, 227). Since 1978 there has been mounting evidence demonstrating that tetracycline use selects for tetracycline resistance. Indeed, the FDA has reported that tetracyclines represented 42% of all antibiotics used in food-producing animals in the US from 2016-2017 (228) and recent data show evidence of the clonal expansion of specific *C. jejuni* genotypes carrying *tet*(O) within cattle isolates. Further, a greater proportion of *C. jejuni* infections in humans have been traced to cattle in the US (229). Thus, surveillance methods should include

mechanisms for monitoring tetracycline resistant isolates to better understand the impact of various selection pressures, HGT, and clonal expansion on the maintenance and dissemination of resistance (230).

CHARACTERIZATION OF *CAMPYLOBACTER*

Culture and molecular typing methods

Campylobacter is generally cultured on antibiotic selective brain-heart infusion (BHI) agar supplemented with rabbit or horse blood at 37^{o}C for 42 – 72hrs in microaerophilic conditions (231). The culture and diagnosis of the disease, however, is not standardized across the US and the use of culture-independent diagnostic tests (CIDT) has become more common. In fact, the increasing frequency of reports of *Campylobacter* over the past decade was thought to be associated with the use of CIDTs (232, 233).

Importantly, several typing methods have also been developed to identify epidemiological and genetic relationships that can aid in outbreak investigation and source attribution as well as pathogen ecology. These typing methods range from phenotypic, fingerprinting, and traditional PCR methods to more sophisticated genotypic methods utilizing whole-genome and pan-genome comparisons (234). Use of these methods has enabled the study of clonal separation and has led to an understanding of the breadth of diversity within *Campylobacter* and other foodborne pathogens. Assessment of these methods typically includes a measure of discriminative power and epidemiological concordance and a unique set of limitations (235, 236).

Serotyping in bacterial populations has been useful to understand the epidemiology of gram-negative pathogens such as non-Typhoidal *Salmonella enterica* spp. and pathogenic *E. coli* as well as gram-positive pathogens. The Kauffman-White scheme, for example, is used to classify *Salmonella* serovars by characterizing the bacterial surface antigens including the O (somatic) antigen and H (flagellar) antigen. This typing scheme was used to make epidemiological inferences about the relationship between particular *Salmonella* serovars and genotypes, which may be due to restricted niches or only sporadic recombination within the species (16, 237–245). In *Campylobacter*, however, different genotypic classifications have been required as these pathogens possess a diverse array of both LOS types as well as capsule types. Therefore, use of a serotyping scheme is less useful for understanding the diversity and epidemiology of *Campylobacter* and *C. jejuni*, in particular.

Fingerprinting methods have also been used to characterize bacterial populations (246). Methods such as pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP), for instance, were historically used for epidemiological surveillance studies for multiple pathogens. For PFGE, rare-cutting restriction enzymes are used to cut the DNA at specific sites in the chromosome followed by gel electrophoresis of the DNA fragments through varied electric fields. This labor-intensive method has been used to identify banding patterns that were alike and aided in epidemiologic and outbreak investigation (247, 248) and is the basis for the CDC PulseNet system (https://www.cdc.gov/pulsenet/pathogens/pfge.html). While PulseNet was used for *C. jejuni*, the analysis was often complicated by the presence of intrachromosomal rearrangements and homopolymeric tracts within the chromosome

(249), which can make two related isolates appear distinct. These regions that are subject to genomic variation enables the pathogen to undergo phase variability within key antigenic structures including the flagella, LOS, and capsule (250–253), which promotes survival and evasion of immune responses (242). Indeed, the success of this pathogen has been attributed to its antigenic diversity as these contingency loci are critical for rapid adaptation in different environments and hosts (254). Because of these cryptic genomic characteristics including homopolymeric tracts and genomic rearrangements, our understanding of the epidemiology and ecology of *C. jejuni* was limited when PFGE and serotyping were used (247).

Several prior reports have shown that independently collected *C. jejuni* isolates rarely had the same PFGE pattern and did not have high concordance with genotyping data generated using multilocus sequencing typing (MLST) (229, 255–262). For MLST, six to eight conserved housekeeping genes have been used to assess the genetic diversity of *Campylobacter* and other foodborne pathogens. Traditionally, these genes, which are roughly 400-600 bp in length, were amplified by PCR, purified, and sequenced. MLST has been described to facilitate the detection of population level changes within a particular pathogen population (263). MLST is particularly advantageous to evaluate the genetic diversity and population structure of *Campylobacter* because it examines a set of conserved genes without evidence of selection (229, 255, 264). Thus, MLST is less likely than PFGE to be impacted by genetic diversity in variable genes that can alter the interpretation of epidemiologic and evolutionary relationships among *Campylobacter* isolates (265–267). Furthermore, extended MLST schemes have been developed that incorporate additional conserved genes such as *gyrA* and *porA,* which encodes for an

outer member protein, into the analysis. These more extensive schemes have demonstrated that both *gyrA* and *porA* are stable across lineages and include sub-lineages that are associated with ciprofloxacin resistance (132, 268).

Pangenome approaches to define the population structure

Today, the availability, ease, and extensive nature of WGS technologies as well as the development of methods to analyze and store data in publicly available databases has improved and availed the investigation of pathogen epidemiology and genomics (229, 231, 269). Tools that enable an assessment of the core-genome (cg), the whole genome (wg), and the pangenome have been utilized to evaluate evolutionary relationships and define the population structure of *Campylobacter* for public health investigation (270). The cg is defined as genes found in most (95 – 99%) isolates. WGS approaches allow for an examination of the entire genome including accessory genes and genes unique to specific strains, within a given bacterial population. Both cgMLST and wgMLST methods have been developed (251, 252), which are based on assigning allele codes to a larger set of housekeeping genes that are shared among a set of strains. These methods provide stability across datasets and enable the assessment and tracking of allele codes across studies for comparison. Use of these approaches, however, requires an international database of curated alleles, and several conflicting schemes for a variety of pathogens have been proposed (259, 271). Although pangenome approaches have been utilized, these approaches do not assign allele codes to cg or wg genes. Genome alignments, *k*-mers or sequence similarity-based methods can be applied to WGS data to better define evolutionary relationships (229, 258–260).

In public health investigations, rigorous analyses to define differences between isolates is critical for source attribution, epidemiological inference, and action during outbreak investigations (271). Currently, cgMLST is utilized for real-time epidemiological inference of foodborne pathogens through the National Center for Biotechnology Institute (NCBI) Pathogen Detection database, however, it is not a publicly available resource. Nextflu and more broadly, NextStrain, are other tools that use molecular clock and iterative phylogenetic placement algorithms, which have enabled real-time genomic epidemiology studies for several pathogens (272). Reference based methods such as cgSNP (271), have also been developed and SNPs are typically detected among all core-genes within a given data set. Reference free SNP methods have also been developed. Lyve-SET, a high-quality SNP (hqSNP) pipeline, for instance, enables a reference free analysis of sequences to better define clonal relationship among isolates, which is particularly useful for closely related strains (273–275). Use of these analytical tools coupled with traditional typing methods such as MLST and PFGE, can yield robust analyses to better define the genomic diversity within a bacterial population and inform public health investigation.

Utilizing pangenome and phylogenomic approaches from publicly available datasets and emerging tools can enable near real-time global genomic epidemiology assessments, which could also be utilized in public health investigations (248, 276–279). Understanding how particular phenotypes or genotypes are restricted to specific environments, or are associated with human exposures, behaviors, and outcomes, could support genotype or exposure specific mitigation strategies (271).

Furthermore, the national molecular subtyping networks, PulseNet and NARMS, allows for high quality genomic information to become more readily available for use by researchers and public health personnel (255, 259, 262). NARMS has begun to use ARG identification and extraction tools, which leverage basic local alignment search tool (BLAST) based algorithms to query assembled contiguous sequences (contigs) (280). Reports have identified concordance with antibiotic resistance genotypes and phenotypes in several pathogens. In *Campylobacter*, phenotypic resistance profiles for several antibiotics were concordant with the presence of specific resistance determinants in prior studies (250, 281). Curated databases by PubMLST (282, 283) and the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) are among the groups that support the use of these databases and the identification of resistance determinants. Using WGS enables genotypic identification that could support genomic epidemiologic inferences including the expansion of specific genetic lineages within a given bacterial population over time.

ECOLOGY AND MOLECULAR EPIDEMIOLOGY

Observations utilizing MLST approaches

Campylobacter is naturally competent and readily takes up DNA from its environment (275). Studies of the *C. jejuni* population structure have identified a diverse pathogen with remarkable evidence of intraspecies recombination (284). MLST analyses of seven loci have identified extensive recombination by applying phylogenetic methods to different parts of the genome. These data have shown that the *Campylobacter* population structure is consistent with the hypothesis that frequent recombination has occurred within the species and suggests that *Campylobacter* does not represent a clonal

population (285). Thus, the frequent recombination and evolutionary history of sequence types (STs) can be studied via placement in a phylogenetic tree, minimum spanning tree, or network to resolve clusters or clonal complexes (CCs) comprised of more closely related strains (286). CCs within *Campylobacter* have been classified based on the clustering of STs relative to the founder genotype, or predominant ST, and the group of derived genotypes that differ in one to three MLST loci (287). Initially, 17 CCs were identified among 814 *C. jejuni* strains (234), though >40 CCs have been described, studied, and reported in PubMLST to date [accessed September 30th, 2021] (287). Of the original 17 CCs, 14 have a central genotype and are distinct from other genotypes, whereas 3 CCs comprising ST-21, ST-48 and ST-206, were described as part of a "super-complex" since they share four of the seven allele sequences (288, 289). The distribution of serotype and *flaA* genotypes that dictate the LOS and H-antigen, respectively, within the MLST-based population structure is dependent on the CC.

Some CCs have a diverse range of serotypes, and other phenotypic characteristics, while CCs including ST-22 and ST-362 are comparatively less diverse (290). Data suggests that natural competency of these derived lineages is important for CC phenotypic diversity as the more phenotypically heterogenous CCs have a higher natural competence quotient (241–243, 291, 292). Additionally, CCs and ST designations have been studied in *Campylobacter* and have ecologic and epidemiologic significance. Shepard et al. (287), for example, utilized a pangenome approach to define a cryptic ecology in the population structure of *C. jejuni* comprising four major lineages. These include two generalist lineages representing strains from multiple sources as well as a cattle and chicken specialist lineage comprising strains solely from each source.

Recombination was present between the cattle and chicken specialist lineages as well as one generalist lineage, but the generalist lineages were found to lack evidence of recombination in nature despite having the ability to recombine as evidenced by *in vitro* studies (287). Specifically, ecological separation was observed as one generalist lineage belonging to CC-45 did not readily recombine and is phylogenetically separated from the other CCs examined (242). Interestingly, ST-45 within CC-45 has been described as a monomorphic lineage of *C. jejuni* that is generally stable and has been detected in different time periods and across sources. Recent data suggest a global distribution of this lineage that was mediated by dispersion through migratory birds (229).

The seven-gene MLST scheme has continued to be relevant in defining the population structure of *C. jejuni* because of the ecologic congruency that enhances the utility of pangenome, and WGS data to attribute these genomic differences to a particular source. These genomic analysis and evolutionary reconstructions can support the identification of emerging lineages, and assessment of antibiotic resistance and virulence determinants in particular populations. Indeed, a few studies have identified specific associations between STs and antibiotic resistance phenotypes such as ST-464 and FQ and tetracycline resistance as well as ST-982 and tetracycline resistance (104, 293). Additional studies have identified clonal expansion of MDR and macrolide resistant isolates in the monomorphic globally dispersed CC ST-45 lineage (289), which can lead to the transfer of resistance determinants to different pathogenic species and other *C. jejuni* lineages.

Pangenome and whole-genome sequencing (WGS) approaches

Prior WGS studies have suggested that *C. jejuni* can adapt to hosts through genome reduction and phase and antigenic variation, while specific mutations have been found to be selected for during human and animal infection (69, 241). Some of these mutations are in “contingency loci” that represent sequences containing insertions or deletions within homopolymeric tracts. Single nucleotide variants (SNVs) within these homopolymeric tracts were suggested to support the rapid diversification of *Campylobacter* by altering expression of phase variable genes encoding antigenic surface structures (59, 60). In the *C. jejuni* genome, the homopolymeric tracts have higher mutation rates with 10 -100 times more mutations than the average of other regions in the genome (292). Due to the intrinsic genomic instability in the homopolymeric tracts, it was suggested that these regions be excluded from public health investigations that use WGS data (292). Exclusion of these regions, from analyses of strains isolated from different environments will ensure that genotypic associations are made with the source of *C. jejuni* and not due to changes that are induced by adaptation to the most recent host (292).

GEOGRAPHIC VARIATION IN *C.JEJUNI* GENOTYPES

More recent studies have used pangenome approaches to define the population structure across geographic locations as well. For example, in Peru, accessory genome diversity differed globally as compared to other representative datasets (294).

The application of MLST has also detected differences in the ST and CC distribution among Michigan patients relative to other geographic locations (104, 293,

295). *C. jejuni* strains belonging to ST-464, for instance, were significantly more likely to be resistant to fluroquinolones (104). Individuals reporting a recent history of foreign travel were also significantly more likely to have infections caused by ST-464 strains. Comparatively, the ST-459 and ST-982 sub-lineages of the “super” CC-21 were most prevalent in strains recovered from cattle (293). ST-982 strains were also more likely to be resistant to tetracycline, which is common among strains recovered from cattle (293), and represented the predominant lineage in Michigan patients, suggesting that cattle may be an important source of *C. jejuni* in Michigan (296). Additional studies using WGS are therefore needed to characterize this pathogen more comprehensively and to identify risk factors for infection and antibiotic resistance.

In 2017, data from New Hampshire using average nucleotide identity and pangenome comparisons as well as an analysis of the core and accessory genome identified diverse phylogenetic lineages of *C. jejuni* that were more related to isolates from other states in the US. These data were used to detect isolates representing ST-2109 that were linked to a multi-state outbreak in puppies between 2016 and 2018 (297).

SUMMARY AND CONCLUSIONS

C. jejuni is a foodborne and zoonotic pathogen with various reservoirs including water, poultry, and ruminants, such as cattle and other livestock (292). Source attribution studies have identified poultry as the major reservoir for *C. jejuni*, where the major pathway of disease transmission in humans is due to consumption of contaminated food products.

Although most cases of sporadic disease in the industrialized world are ascribed to contaminated poultry products, consumption of raw or unpasteurized milk or cheese was reported to comprise 66% of outbreaks associated with *C. jejuni* (176, 179). Similarly, cattle were suggested to be an important source of *C. jejuni* infections in a prior Michigan study (293). Foreign travel has also been reported as an important risk factor for disease, as zoonotic, foodborne, and environmental acquisition are the major modes of transmission for this pathogen (213, 214). State, national, and global surveillance of campylobacteriosis suggests an increase in the incidence over time, which could be due in part to the use of CIDTs in clinical laboratories or increasing frequencies of antibiotic resistant strains that are more difficult to treat (295).

Monitoring antibiotic resistance in *C. jejuni* is also critical as the CDC has identified macrolide and FQ resistant *Campylobacter* as significant threats to public health (204). Of concern is the stability and relative maintenance of FQ resistance even in the absence of selection through FQ use. Recent data suggest a larger proportion of campylobacteriosis in the US is being attributed to cattle, which was suggested to be due to the consumption of contaminated dairy products (176, 179). FQ resistant *C. jejuni* in domesticated cattle and animal products could therefore contribute to the stability of the pathogen in the agro-environment and the increase in FQ resistance frequencies observed regionally and nationally (213, 214).

The use of WGS to identify associations between specific *C. jejuni* lineages and phenotypic and epidemiologic characteristics is critical to better understand this genetically diverse pathogen. Such analyses could impact applied epidemiologic studies and aid in the development of novel mitigation strategies in the future with a goal of limiting

C. jejuni transmission and improving morbidity and mortality. Examples of strategies include preventing point-source and diffuse outbreaks and reducing practices that promote the selection of antibiotic resistant strains that are harder to treat and may have more severe outcomes (125).

Understanding global, national, regional, state, and territorial trends in antibiotic resistance frequencies is complex. Resistance frequencies will be impacted by an array of factors including selection pressure and fitness effects of resistance determinants including the ability of resistant lineages to replace antibiotic susceptible lineages in a niche. Additionally, frequencies of resistance will be impacted by human behavior as exposures change and new risk factors are identified. FQ resistant *C. jejuni* frequencies may be impacted by the annual rates of cattle- or travel associated infections, for instance. Such changes could explain the persistence and increasing frequencies of FQ resistance in the US. An assessment of risk-factors, frequencies, and impacts of resistance on clinical outcomes are also critically important for surveillance, prevention, and mitigation strategies. Understanding the genomic epidemiology and structure of the bacterial population across studies is also important as is the analysis of how specific lineages are related to phenotypes and epidemiologic factors. These studies will enable an assessment of clonal expansion, emerging lineages, and lineage associations with antibiotic resistance phenotypes.

Linkage of these data can support public health investigations and has the potential to influence decision making as well as mitigation and eradication strategies. Ultimately, use of these highly discriminative methods will be important for public health and outbreak investigations, global genomic surveillance, and pangenome analyses.

WGS approaches could also enhance understanding of niche specialization and further identify phenotypic, epidemiologic and host associations for *C. jejuni.*

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CHAPTER 2: EPIDEMIOLOGIC ASSOCIATIONS VARY BETWEEN TETRACYCLINE AND FLUOROQUINOLONE RESISTANT *CAMPYLOBACTER JEJUNI* INEFCTIONS

PREFACE

The characterization of antibiotic resistance and epidemiologic associations of Campylobacter jejuni *have been presented in this chapter has been previously published:*

Rodrigues JA, Cha W, Mosci RE, Mukherjee S, Newton DW, Lephart P, Salimnia H, Khalife W, Rudrik JT, Manning SD. 2021. Epidemiologic Associations Vary Between Tetracycline and Fluoroquinolone Resistant *Campylobacter jejuni* Infections. *Front Public Heal* 9:672473.

Author Contributions Statement: SDM, WC and JAR designed the study; JTR, DN HS, PL, and WK organized sample collection at each site; REM organized samples and extracted the epidemiological data; JAR and WC performed the experiments; JAR, SM and SDM managed the data and conducted analyses; and JAR developed the first manuscript draft. All authors contributed and approved the manuscript content.

ABSTRACT

Campylobacter jejuni is the leading cause of bacterial gastroenteritis and antibiotic resistant *C. jejuni* are a serious threat to public health. Herein, we sought to evaluate trends in *C. jejuni* infections, quantify resistance frequencies, and identify epidemiological factors associated with infection. *C. jejuni* isolates (n=214) were collected from patients via an active surveillance system at four metropolitan hospitals in Michigan between 2011 and 2014. The minimum inhibitory concentration for nine antibiotics was determined using microbroth dilution, while demographic and clinical data were used for the univariate and

multivariate analyses. Over the four-year period, a significant increase in the recovery of *C. jejuni* was observed ($p \leq 0.0001$). Differences in infection rates were observed by hospital and several factors were linked to more severe disease. Patients residing in urban areas, for instance, were significantly more likely to be hospitalized than rural residents as were patients over 40 years of age and those self-identifying as non-White, highlighting potential disparities in disease outcomes. Among the 214 *C. jejuni* isolates, 135 (63.1%) were resistant to at least one antibiotic. Resistance was observed for all nine antibiotics tested yielding 11 distinct resistance phenotypes. Tetracycline resistance predominated (n=120; 56.1%) followed by resistance to ciprofloxacin (n= 49; 22.9%), which increased from 15.6% in 2011 to 25.0% in 2014. Resistance to two antibiotic classes was observed in 38 (17.8%) isolates, while multidrug resistance, or resistance to three or more classes, was observed in four (1.9%). Notably, patients with ciprofloxacin resistant infections were more likely to report traveling in the past month (Odds Ratio (OR): 3.0; 95% confidence interval (CI): 1.37, 6.68) and international travel (OR: 9.8; 95% CI: 3.69, 26.09). Relative to patients with only tetracycline resistant infections, those with ciprofloxacin resistance were more likely to travel internationally, be hospitalized and have an infection during the fall or summer. Together, these findings show increasing rates of infection and resistance and highlight specific factors that impact both outcomes. Enhancing understanding of factors linked to *C. jejuni* resistance and more severe infections is critical for disease prevention, particularly since many clinical laboratories have switched to the use of culture-independent tests for the detection of *Campylobacter*.

INTRODUCTION

Campylobacter spp. are a leading cause of bacterial gastroenteritis infections worldwide (1) and represent the most common cause of foodborne infections in the U.S. since 2013 (2). While *C. jejuni* causes a vast majority of human infections, other species including *C. coli*, *C. upsaliensis*, *C. lari*, *C. fetus*, *C. insulaeingrae*, and *C. hyointestinalis*, are also important (3). Collectively, these pathogens were estimated to cause 1.5 million infections in the U.S. each year, contributing to 13,000 hospitalizations and 120 deaths (4). In 2018, the Centers for Disease Control and Prevention (CDC) estimated the incidence of campylobacteriosis to be 19.6 cases per 100,000 individuals, which had increased from 12.0 cases per 100,000 in 2015-2017, among sites participating in the Foodborne Diseases Active Surveillance Network (FoodNet) (2).

Clinical manifestations of campylobacteriosis include fever, abdominal pain, vomiting, weight loss, chills, fatigue, myalgia, malaise, and acute watery or bloody diarrhea (1). The incubation period is typically one to four days after exposure, and the severity of symptoms tends to vary by bacterial density and strain (5). Post-infectious immune sequalae such as Guillain-Barré Syndrome, Miller-Fisher syndrome, and reactive arthritis, have been linked to *Campylobacter* infection as have inflammatory bowel disease, esophageal and colo-rectal cancers, and extra-intestinal infections like bacteremia and meningitis (6). Although most infections are self-limiting, antibiotics are often needed for immunocompromised patients or those with more severe or persistent infections (7).

Water, poultry, and livestock are common reservoirs for *C. jejuni* (8). Transmission to humans typically occurs via consumption of contaminated food products, and direct

contact with animal or environmental reservoirs (9). According to a meta-analysis of 72 studies, the key risk factor for campylobacteriosis was international travel, yet consumption of undercooked chicken and direct exposure to *Campylobacter* from the environment or farm animals were also important (10). Regardless, it is important to note that risk factors often vary by geographic location even across the U.S., with different FoodNet sites reporting considerable variation in the frequency of infections (11). In addition, the FoodNet sites were not selected to be representative of the U.S. population and were shown to have an unequal representation of all racial and ethnic groups and contained fewer individuals living below the poverty level (12, 13).

C. jejuni has also been designated a serious antibiotic resistant threat resulting in 448,400 resistant infections and 70 deaths each year (14). Resistance to ciprofloxacin, a fluoroquinolone used to treat more severe human infections, increased in the U.S. from 13% in 1997 to 25.3% in 2015 (14, 15). *C. jejuni* resistance to multiple drug classes has also increased over time (16) and resistant isolates have been linked to more severe infections requiring lengthier hospitalizations (17). Because NARMS does not utilize data from each state and the Midwest region only receives a subset of *Campylobacter* isolates from the Minnesota FoodNet site for testing (18), these resistance frequencies and trends may not be representative of those in other locations. Additionally, many clinical laboratories have shifted to the use of culture-independent tests to detect *Campylobacter* infections, which can obscure actual rates of resistance circulating within patient populations and prevent the identification of risk factors for resistant infections. Indeed, a 2019 FoodNet report noted that 42% of *Campylobacter* infections were detected using a culture-independent test (2). This shift is concerning and highlights the need for more

culture-based studies to better define the epidemiology of and resistance phenotypes in this common foodborne pathogen.

Herein, we sought to describe the susceptibility profiles for 214 *C. jejuni* isolates cultured from patients with campylobacteriosis during surveillance activities in Michigan (2011-2014) and to identify risk factors for both susceptible and resistant infections. We also sought to make comparisons to national data available through NARMS since *Campylobacter* resistance is not monitored in Michigan via NARMS (19). Studies such as these highlight the importance of using culture-based diagnostic tests to more accurately monitor resistance phenotypes and frequencies in distinct geographic locations to identify potential exposures and risk factors that may be state and/or region specific.

MATERIALS AND METHODS

Strain source and speciation

Campylobacter isolates were recovered from stools of patients with campylobacteriosis between 2011 and 2014 via an active surveillance system at four metropolitan hospitals located in Detroit, Grand Rapids, Ann Arbor, and Lansing, Michigan. Isolates were transported to the Michigan Department of Health and Human Services (MDHHS) and stored in 10% skim milk at -80°C until use.

Isolates were thawed and cultured on Tryptone Soy Agar (TSA) containing 5% sheep blood and cefoperazone (20 µg), amphotericin B (4µg/mL), and vancomycin (20µg/mL) in microaerophilic conditions (20). DNA was extracted and multiplex PCR was performed to classify the species of each *Campylobacter* isolate using a previously described protocol (21). Briefly, the Kapa2G Taq (Kapa Biosystems; Wilmington, MA)

was used for PCR amplification using the following conditions: denaturation at 95°C for 15 minutes followed by 25 cycles of 95°C for 30 seconds and 58°C for 1 minute and 30 seconds and 72°C for 8 minutes. Roughly 91 of the 214 (43%) *C. jejuni* isolates included in the analysis were characterized previously (22).

Antimicrobial susceptibility profiling

The minimum inhibitory concentration (MIC) was determined for nine antibiotics using microbroth dilution utilizing Sensititre™ *Campylobacter* Campy AST plates (ThermoFisher; Waltham, MA) according to the manufacturer's protocols. The antibiotics (classes) were: ciprofloxacin (fluoroquinolone), nalidixic acid (quinolone), azithromycin (macrolide), erythromycin (macrolide), tetracycline, florfenicol (phenicol), telithromycin (ketolide), clindamycin (lincomycin), and gentamicin (aminoglycoside). *C. jejuni* ATCC 33560 was used a control. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards were used, as per the current NARMS protocol, for classifying isolates as resistant or susceptible (19). NARMS data were extracted from isolates collected in the same time period for comparison. Isolates with any ciprofloxacin resistance and any tetracycline resistance were counted; these two categories were not mutually exclusive as some isolates had resistance to both drugs. A subset of data submitted from NARMS Region 5 representing the Midwest (Ohio, Indiana, Michigan, Illinois, Wisconsin, and Minnesota and 34 federally recognized tribes) were also included in this analysis for comparison.

Epidemiological variables and data analysis

Demographics, exposures, and clinical data were extracted from the Michigan Disease Surveillance System (MDSS), an online database containing epidemiological data for notifiable infections. The sample collection date was used to classify the season as follows: spring (March, April, May), summer (June, July August), fall (September, October, and November), and winter (December, January, and February). Cases reporting a history of travel in the past month were classified as traveling domestically (within the U.S.) or internationally. Michigan counties were designated as urban or rural based on data presented in a National Center for Health Statistics report (23); all but ten Michigan counties were considered rural. Cattle densities per county were obtained from a 2019 report (United States Department of Agriculture, 2020), and the high versus low categories were developed based on the average number of cattle in all Michigan herds with data available.

Chi-square tests were used for dichotomous variables to identify associations between the dependent and independent variables, while the Mantel-Haenszel Chi-square test was used to examine trends. Differences in proportions were evaluated using the Chi-square test for equal proportions and for variables with small sample sizes, or less than five per cell, the Fisher's exact test was used. A p-value ≤ 0.05 was considered significant for each test, however, all variables yielding a p-value ≤ 0.20 in the univariate analysis were included in the multivariate analyses. Potential confounders such as age, sex, and residence location, were also included in the forward logistic regression analyses to identify predictors of each outcome. Odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated to describe the magnitude of each association. SAS

version 9.4 (SAS Institute, Cary, NC, USA) and Epi Info™ version 7 were used. All protocols were approved by the Institutional Review Boards at Michigan State University (10-736SM), the MDHHS (842-PHALAB) and each participating hospital.

RESULTS

Recovery of *Campylobacter* in Michigan, 2011-2014

In all, 277 *Campylobacter* isolates were recovered from Michigan residents diagnosed with campylobacteriosis at four large metropolitan hospitals between January 2011 and December 2014. Approximately 234 (84.5%) of the isolates were viable and could be speciated using PCR. Among these, 217 (92.7%) were classified as *C. jejuni*, while fifteen (6.0%) were *C. coli*; two isolates (0.9%) were characterized as *C. upsaliensis*. Given that *C. jejuni* was the most common species, the analysis was restricted to these isolates and cases. Three additional isolates from residents living outside of Michigan were also excluded from the analysis.

Significant variation in the recovery of *C. jejuni* was observed across hospitals ($p \leq 0.0001$), with most isolates (n=174; 82.1%) coming from two sites; the hospital location was missing for three isolates. The frequency of *C. jejuni* at each site was 42.9% (n=91), 39.2% (n=83), 7.6% (n=16), and 9.9% (n=21). A significant difference in the recovery of *C. jejuni* isolates was also observed over time with 57.5% (n=123) of the infections occurring in 2013 and 2014 ($p \leq 0.0001$). Differences were also observed by season since more isolates were recovered in the summer and fall months (n=158; 73.8%) compared to the winter and spring (n=56; 26.2%) ($p \leq 0.0001$). Moreover, a greater proportion of cases resided in urban (n=119; 62.0%) versus rural (n=73; 38.0%) areas ($p = 0.0009$).

Demographics and exposure history of *C. jejuni* cases

Among the 214 *C. jejuni* cases from Michigan residents, 110 (54.4%) were male and 53.1% (n=113) were between the age of 19 and 65 years; the age was missing for one case **(Table 2.1)**. Sixty-five (30.5%) cases represented children between one day and nine years of age. Twenty (30.8%) of these children were ≤1 year old and 33 (50.8%) were between 1 and 5 years of age. Among the 17 elderly patients over 65 years, over half (n=11) were between 70 and 87 years of age. Significantly more cases self-identified as White/Caucasian (n=137; 79.7%), though a subset self-identified as Black/African American (n=17; 9.8%), Asian (n=6; 3.5%), or another race (n=13; 7.5%). Thirteen (8.8%) cases self-identified as Hispanic/Latino and 25 (19.1%) self-identified as Arab, however, up to 83 (38.8%) cases did not indicate their ethnicity.

The majority (n=88; 59.9%) of cases did not travel in the month prior to infection compared to 40.9% of cases who did. Among 59 of the 61 cases reporting their travel location, 18.4% (n=27) traveled internationally and 22.4% (n=33) reported domestic travel. While more of these cases traveled during the summer (n=30; 44.4%) and fall (n=18; 29.5%) as opposed to the winter and spring (n=13; 21.3%), the difference was not significant (p = 0.26).

Despite the greater proportion of cases reporting animal contact prior to illness onset (n=96; 64.0%), multiple animal species were reported. Among these cases, 88 (91.7%) cases reported contact with domestic animals, 13 (13.5%) with livestock and 11 (11.5%) with birds or poultry. Seventeen cases (17.7%) reported contact with other animals and three (3.1%) had contact with reptiles. Furthermore, most (n=199; 81.5%) cases drank municipal and/or bottled water and consumed poultry (n=115; 87.8%) up to

a week prior to symptom onset. Food history data, however, was not available for up to 38.8% of the cases.

Because significantly more cases lived in urban areas, we also sought to determine whether any factors were associated with urban residence (**Table 2.2**). Importantly, the odds of hospitalization for urban residents was significantly greater (n =34; 73.9%) than rural residents (n=12; 26.1%), yet no differences in symptoms were observed. Urban patients were also significantly less likely to report any travel in the past month, either domestic or international, and were less likely to be between 19 and 40 years of age than rural patients. Indeed, 83.6% (n=46) of children less than 10 years of age resided in an urban area compared to 16.4% (n=9) for rural children. Although exposure to livestock and Arab ethnicity were significantly associated with rural and urban residence, respectively, the sample sizes were small for each variable and many records had missing data.

Clinical symptoms and association with more severe infections

Among the subset of cases reporting symptoms, diarrhea (95.0%) was the most common followed by abdominal pain (69.7%), nausea (41.3%), and fatigue (40.9%). Only 36.2% of cases reported the presence of bloody diarrhea, while 34.5%, 28.7%, and 26.6% reported chills, body aches, and headaches, respectively. In all, 46 (25.3%) patients were hospitalized ranging from one day to 11 days with an average of three days.

In addition to urban residence, several other factors were associated with hospitalization, a marker for more severe disease, in the univariate analysis (**Table 2.3**). An increasing odds of hospitalization was observed as age increased. Compared to

adults between years, adult patients between 41 and 65 years and the elderly over 65 years were significantly more likely to be hospitalized. The same was true when children less than 9 years was used as the reference group. Patients self-reporting nausea and fatigue were also more likely to be hospitalized as were patients self-identifying as non-White. By contrast, patients reporting domestic or international travel in the month prior to symptom onset were significantly less likely to be hospitalized. No association was observed for sex, season, source of drinking water, or any other symptoms, and no differences were detected when the analysis was limited to only those individuals without a recent history of international travel.

Controlling for potential confounders such as residence type (urban versus rural), sex, season, and age, multinomial logistic regression identified the oldest age groups, 41-65 years (adjusted OR: 6.1; 95 CI: 2.37, 15.70) and >65 years (adjusted OR: 10.5; 95 CI: 2.63, 42.19), to be predictors of hospitalization relative to the younger age groups. International travel in the past month (adjusted OR: 0.3; 95% CI: 0.07,0.94), non-White race (adjusted OR: 4.8; 95% CI: 1.62, 14.01), and nausea (adjusted OR: 2.8; 95% CI: 1.15, 6.68) were also independently associated with hospitalization.

Antibiotic resistance phenotypes and frequencies

Resistance was detected in 63.1% (n=135) of the 214 *C. jejuni* isolates and at least one isolate was resistant to each of the nine antibiotics tested. Tetracycline resistance (n=120; 56.1%) predominated followed by resistance to ciprofloxacin (n=49; 22.9%) **(Figure 2.1A).** Fewer than five isolates had resistance to clindamycin, azithromycin, and telithromycin, and only one isolate was resistant to gentamicin and another to phenicol.

All isolates with ciprofloxacin resistance were also resistant to nalidixic acid. Among the 135 resistant isolates, 93 (43.5%) were resistant to one class of antibiotics, whereas 38 (17.8%) were resistant to two. In all, 11 different *C. jejuni* resistance phenotypes that varied in frequency **(Figure 2.1B**). Five of these phenotypes included tetracycline resistance, six included ciprofloxacin resistance, and three phenotypes included both. The predominant phenotypes were tetracycline resistance alone (n=82; 38.3%) and in combination with ciprofloxacin (n=35; 16.4%). Multidrug resistance (MDR), which is defined as resistance to three or more antibiotic classes, was observed in four (1.9%) isolates.

Fluctuations in resistance frequencies were observed by year. Although no significant increase in any resistance or MDR was observed over the four-year period, notable trends were observed for some phenotypes **(Figure 2.2)**. For instance, a significant decrease in the frequency of isolates with only tetracycline resistance was observed over time (p = 0.04), while a slight insignificant increase in ciprofloxacin resistance was observed alone ($p \leq 0.24$) and in combination with tetracycline resistance ($p \leq 0.50$). Despite the gradual increase in the frequency of any resistance to ciprofloxacin from 15.6% in 2011 to 25.0% in 2014, the change was not significant ($p \leq 0.31$). The same was true for any resistance to tetracycline, which decreased from 65.6% in 2011 to 52.5% in 2014 (p = 0.17).

Epidemiological associations with antibiotic resistant *C. jejuni* infections

Several notable associations were identified between epidemiological factors and the most common antibiotic resistant phenotypes, ciprofloxacin resistance and

tetracycline resistance. These two predominant phenotypes were classified as the dependent variables to uncover associations with each phenotype relative to cases with either susceptible infections or infections with resistance to all other antibiotics.

Patients with ciprofloxacin resistance (n=49) were more likely to travel in the month prior to infection (OR: 3.0; 95% CI:1.37, 6.68) relative to all other cases (**Table 2.4**). More specifically, they were more likely to report international travel (OR:9.8; 95% CI:3.69, 26.09).Patients with tetracycline resistance (n=120) were also more likely to report international travel in the past month, however, the difference was not significant (OR: 2.2; 95% CI:0.85, 5.46). Patients with tetracycline resistance were also significantly less likely to have an infection during the summer or fall months (OR: 0.5; 95% CI:0.27, 0.97) and to report contact with livestock (Fisher's exact test p=0.04) or well water (OR: 2.3; 95% CI:0.95, 5.75); the latter association was not significant. No association was observed between resistance to either antibiotic and domestic travel history, hospitalization, or clinical symptoms including body aches, diarrhea with blood, fatigue, fever, abdominal pain, and headache.

Because a subset of the isolates had both tetracycline and ciprofloxacin resistance, we also created mutually exclusive categories to identify risk factors for each. In this analysis, ciprofloxacin resistance was defined as any resistance to ciprofloxacin even if resistance to other drugs including tetracycline was observed. Among the 135 resistant isolates, 49 (36.3%) had ciprofloxacin resistance. Tetracycline resistance was defined as any resistance to tetracycline but without the co-occurrence of ciprofloxacin resistance; 83 (61.5%) isolates had tetracycline resistance without ciprofloxacin resistance. Individuals with susceptible isolates and those representing different

resistance profiles were excluded from the analysis. Compared to patients with tetracycline resistance, those with ciprofloxacin resistant infections were significantly more likely to report traveling in the past month (OR: 2.9; 95% CI:1.20, 7.02) and specifically, international travel (Fisher's exact test p <0.0001) (**Table 2.5**). Only four (19.1%) of the 21 patients who traveled internationally had tetracycline resistant infections compared to 17 (81.0%) of those with ciprofloxacin resistant infections. A difference was also observed for hospitalization, which was significantly more common in ciprofloxacin resistant infections (OR: 2.5, 95% CI:1.02, 6.14), while contact with livestock was more common in tetracycline resistant infections (Fisher's exact test p = 0.09), yet the latter association was not significant. No association was observed for age, sex, race, ethnicity, residence types, season, water source, cattle density, poultry consumption, or clinical symptoms.

Multinomial logistic regression was performed to identify predictors of ciprofloxacin resistance relative to tetracycline resistance while controlling for age, sex, urban residence, season, and international travel. Notably, international travel in the past month (adjusted OR: 13.0; 95% CI: 3.71, 45.64) and infection during the summer or fall months were the only significant predictors of ciprofloxacin resistance (**Table 2.5**). Hospitalization was also more common in patients with ciprofloxacin resistance than tetracycline resistance, yet the association was not significant in the model (adjusted OR: 2.4; 95% CI: 0.86, 6.46), which could be due to the small sample size.

Comparing resistance frequencies to national data reported via NARMS

A comparison of resistance frequencies for the NARMS isolates recovered during the same time period uncovered region-specific differences for both ciprofloxacin and

tetracycline resistance. A significantly greater proportion of the Michigan isolates were resistant to tetracycline when compared to the 3,457 isolates from all regions except Region 5. Although Region 5 covers Michigan and other midwestern states, the data were generated by examining only a subset of isolates recovered from the Minnesota FoodNet site (**Figure 2.3A**). No difference in tetracycline resistance frequencies was observed between Michigan and the Region 5 isolates (n=585), or for any of the ciprofloxacin resistance frequencies. However, when our Michigan isolates (n=214) were added to Region 5, differences were observed for both ciprofloxacin and tetracycline resistance across the NARMS regions (**Figure 2.3B**). Notably, Region 5 had significantly more tetracycline resistance than all other regions combined (OR: 1.6; 95% CI: 1.41, 1.92) as well as a significantly greater proportion of ciprofloxacin resistance than Regions 2 (OR: 1.6; 95% CI: 1.24, 2.03) and 6 (OR: 2.1; 95% CI: 1.50, 3.00). Relative to Region 1, however, the proportion of ciprofloxacin resistance was significantly lower than in Region 5 (OR: 0.6; 95% CI: 0.50, 0.79). No other differences were observed for ciprofloxacin resistance by region.

DISCUSSION

Through this study we have detected important trends in the prevalence of campylobacteriosis and antibiotic resistant *C. jejuni* isolated from Michigan patients between 2011 and 2014, further highlighting the importance of pathogen surveillance efforts using culture-based methods. Since campylobacteriosis was not classified as a notifiable infection until 2015 (25), data about disease frequencies and resistance profiles have been limited, particularly for states like Michigan that are not participating in FoodNet

or NARMS. In addition, the widespread adoption of culture-independent tests has hampered the ability to routinely monitor important phenotypes such as antibiotic susceptibility profiles. Indeed, it was estimated that 42% of campylobacteriosis cases identified via FoodNet in 2019 were diagnosed by culture-independent tests and among these, culture for *Campylobacter* was only attempted for 63% of the positive samples (2).

In the four Michigan hospitals examined herein, we observed a significant increase in *C. jejuni* infections over time, which is similar to national trends (2) and could partly be due to improved sampling and detection capacity. Seasonal differences were also observed with a greater proportion (73.8%) of Michigan cases occurring during the summer and fall. Seasonal variation has been reported previously with several studies showing a peak incidence of *C. jejuni* infections during warmer months; climate, temperature, increased shedding from animal reservoirs, and/or seasonal-specific behaviors have all been suggested to contribute to seasonality (26–28).

Extracting epidemiological data from case records has also facilitated the identification of factors that increase risk of campylobacteriosis. Similar to our prior study of 7,182 campylobacteriosis cases reported in Michigan between 2004 and 2013 (29) and those from the FoodNet sites (25, 30), most infections affected children <10 (30.8%) or adults between 19-65 (53.1%) years of age. Despite this bimodal distribution, the likelihood of hospitalization increased with increasing age. Cases between 41 and 65 years were significantly more likely to be hospitalized than those between 19 and 40 years of age as were cases over 65. The link between older age and more severe disease has been reported for the FoodNet sites and in our prior population-based study (29, 31).

Although males and rural residents represented a greater proportion of the 7,182 campylobacteriosis cases in Michigan (29), similar distributions were not observed among the cases at the four hospitals. For instance, no difference was observed by sex and significantly more cases (62.0%) were from urban areas, suggesting that the four hospitals may not be entirely representative of the Michigan population of campylobacteriosis cases. Such differences are likely due to the structure of the surveillance system since we utilized four of the largest health care systems. Despite having wide catchment areas, each hospital is in a metropolitan location that can result in differences in access to health care, particularly for rural residents, supporting the suggestion that geography as well as patient-specific and cultural factors can impact care seeking behaviors (11). Indeed, we observed a lower likelihood of hospitalization among rural residents in this and our prior study (29), although this association was not significant after controlling for race, sex, and age.

Because the likelihood of hospitalization was significantly greater for cases self-identifying as non-White, urban areas should be an important focus for reducing disparities in infections caused by *C. jejuni* and other enteric pathogens. Certainly, neighborhood and geographic barriers have previously been suggested to be important for the acquisition of foodborne disease (12). Although race, ethnicity, and other socially constructed categorizations such as socio-economic status, are not typically collected for foodborne disease surveillance systems, prior studies have document increased frequencies of gastroenteritis in minority and low-socioeconomic populations globally (32–34). Additional studies are needed, however, to identify specific risk factors, exposures and causal factors within urban environments that may explain these

relationships. Use of previously reported proxies and markers of poverty such as urban residence, and social constructs like self-reported race as we have used, have complex interactions with social determinants of health (35, 36). We therefore cannot describe causal factors for hospitalization of *C. jejuni* without addressing these shortcomings. We also cannot rule out the possibility that different strain populations with distinct pathogenic traits are circulating in the different areas and are partly responsible for the differences observed.

Since most hospital laboratories in Michigan have switched to the use of culture-independent tests to detect *C. jejuni*, viable isolates are not typically recovered for characterizing important phenotypic or genotypic traits. Hence, our assessment of resistance frequencies and trends in the four hospitals over this four-year period yielded notable results. Resistance was detected in 63.1% of the 214 isolates and to all nine antibiotics comprising 11 distinct resistance profiles. The overall predominance of tetracycline (56.1%) and ciprofloxacin (22.9%) resistance was similar in our prior study of 94 isolates recovered in 2011 and 2012 (22). The inclusion of 120 additional isolates recovered from the same hospitals in 2013-2014, however, allowed for the detection of several important changes over time, including an increase in the frequency of fluoroquinolone resistance. This gradual increase is concerning given that fluoroquinolones are commonly used to treat human infections and the Food and Drug Administration (FDA) banned use of these drugs in poultry in 2005 (37). Point mutations in chromosomal genes such as *gyrA*, which is critical for DNA replication and transcription, have been linked to fluoroquinolone resistance (38, 39). Given that these mutations do not halt transcription, there is no impact on bacterial survival and hence,

these resistant bacterial populations can persist in the absence of antibiotic selection (40, 41). This increasing frequency of ciprofloxacin resistance in *C. jejuni* is consistent with national trends for older strain sets recovered via culture-based detection methods (25). Because of the increased use of culture-independent methods to detect *Campylobacter*, however, actual rates of ciprofloxacin resistance among clinical isolates in different parts of the U.S. are not well established. Additionally, despite the critical role that FoodNet and NARMS have played in the detection of resistant foodborne pathogens, neither system is entirely representative of the U.S. population (Hardnett et al., 2004). To represent the entire Midwest (Region 5) that includes Michigan, for instance, NARMS only receives a subset of isolates from Minnesota for testing (18). It is therefore important to note that the frequencies and trends reported by NARMS may not accurately reflect those observed in other locations with distinct geographic features and population traits.

Furthermore, fluoroquinolone resistant *C. jejuni* infections have also been reported to increase the duration of illness (42, 43). Mutations in *gyrA* have been tied to changes in DNA supercoiling, which can lead to enhanced colonization of the chicken gut and an increase in virulence properties such as motility, biofilm formation and invasion of intestinal epithelial cells *in vitro* (44–47). These studies establish a mechanism by which fluoroquinolone resistant mutations enhance virulence and support prior associations between resistance and a lengthier illness duration. Additional support comes from our finding that patients with ciprofloxacin resistant infections were twice as likely to be hospitalized than patients with tetracycline resistant infections, suggesting that the former may be more severe. It is not clear, however, if a unique patient population, differential

treatment regimens, or distinct bacterial factors account for the difference in the hospitalization rates observed.

Significant differences in the frequency of tetracycline resistance, which was highest in this population of Michigan patients despite the gradual decrease in tetracycline resistance over time, were also observed. These data indicate that unique regional factors may impact resistance rates, yet those factors that contribute to variation across locations are not clear. The tetracyclines have been used to treat zoonotic and rickettsial diseases in human medicine (48) and have been used extensively in livestock and poultry production worldwide. In the U.S., the FDA reported that tetracyclines were the predominant drug class used in food-producing animals at the time of this study (2009-2014), representing an average of 42% of all antibiotics used (49). Continuous use of tetracycline has selected for resistant strains and resistance genes that can persist in reservoir hosts and the environment. For example, TetO has been shown to mediate resistance to tetracycline in *C. jejuni* by offering ribosomal protection by binding to an unoccupied site (39). This protein is encoded by *tet*(O), which is commonly carried on the pTet plasmid but has also been detected in the chromosome (50, 51). Given the high transmissibility rates of these resistance plasmids within bacterial populations even in the absence of tetracycline use (52, 53), it is clear that *C. jejuni* serves as an important reservoir for these and other resistance genes. In our prior study, we demonstrated that tetracycline resistance was more common in strains belonging to multilocus sequence type (ST)-982, a lineage that was also common in Michigan cattle (22, 54) and has been linked to livestock in other locations (55, 56). Together, these data show the importance

of clonal expansion of resistant lineages and highlight the role that mobile genetic elements can play in dispersion and maintenance of tetracycline resistance.

Although we observed a significant association between livestock contact and tetracycline resistance, the number of cases (n=13) reporting this exposure was low. It is noteworthy, however, that only one of the cases reporting contact with livestock had a ciprofloxacin resistant infection compared to 11 (84.6%) with tetracycline resistant infections. While increasing frequencies of ciprofloxacin resistant *C. jejuni* have been recovered from feedlot cattle throughout the U.S. (57), our data suggest that different factors are important for the acquisition of ciprofloxacin versus tetracycline resistant infections. Consistent with prior studies (25, 30, 42, 58), we have demonstrated that international travel in the month prior to infection is the strongest predictor of ciprofloxacin resistance in this sample of Michigan patients. Infection during the summer or fall months was also independently associated with ciprofloxacin resistance, but we did not observe an association with poultry consumption as was described in other studies (42, 58, 59). This difference could be due to the low number of cases reporting no poultry consumption a week before symptom onset or the high frequency of missing data since many patients failed to answer the food history questions, a common problem with long-term epidemiological studies (60). In general, however, the identification of risk factors that have also been described in other studies is encouraging and indicates that these factors are likely important regardless of the geographic location.

Collectively, the data presented herein highlight the importance of monitoring antibiotic resistance phenotypes and frequencies using culture-based methods in multiple geographic locations. The significant difference that we observed in NARMS Region 5

relative to other regions after including our Michigan data with those from Minnesota, illustrates the need for more comprehensive testing and highlights the variation across different geographic locations. Future studies are still needed, however, to link resistance profiles and patient data to epidemiological data to identify those exposures and risk factors that are unique to specific states or regions.

APPENDIX

Table 2.1 Differences in the proportion of specific characteristics among 214 cases infected with *Campylobacter jejuni* in Michigan, 2011-2014.

Case characteristics	No. [a] (%)	Chi square [b]	DF	*p* value
Age (years)		50.7	4	<0.0001
0-9	65 (30.5)			
10-18	18 (8.5)			
19-40	54 (25.4)			
41-65	59 (27.7)			
>65	17 (8.0)			
Sex		0.95	1	0.33
Male	110 (53.4)			
Female	96 (46.6)			
Self-reported race [c]		275.4	3	<0.0001
White/Caucasian	137 (79.7)			
Black/African American	17 (9.9)			
Asian	6 (3.5)			
Other	12 (7.0)			
Self-reported Hispanic ethnicity		99.6	1	<0.0001
Non-Hispanic/Latino	134 (91.2)			
Hispanic/Latino	13 (8.8)			
Self-reported Arab ethnicity		50.1	1	<0.0001
Non-Arab	106 (80.9)			
Arab	25 (19.1)			
County classification		11.0	1	0.0009
Rural	73 (38.0)			
Urban	119 (62.0)			
Cattle density in resident county[d]		33.1	1	<0.0001
Low (<8400 cattle)	23 (21.9)			
High (≥8400 cattle)	82 (78.1)			
Any travel in the past month		4.9	1	0.03
No	88 (59.1)			
Yes	61 (40.9)			
Type of travel in the past month		46.8	2	<0.0001
None	88 (59.9)			
Domestic travel only	33 (22.4)			
Any international travel	27 (18.4)			

Table 2.1 (cont'd)

Animal contact			11.8	1	0.0006
No	54	(36.0)			
Yes	96	(64.0)			
Poultry consumption in the past week			74.8	1	<0.0001
No	16	(12.2)			
Yes	115	(87.8%)			
Drinking water			58.0	1	<0.0001
Municipal, bottled	119	(81.5)			
Any well water	27	(18.5)			

[a] Not all numbers (No.) add up to the total number of 214 cases due to missing data for some variables.
[b] Significant differences were identified using the Chi-square Test for Equal Proportions; DF = degree of freedom.
[c] Self-reported race categories in the online Michigan Disease Surveillance System questionnaire were Caucasian, African American, Asian, American Indian/Alaska Native, Hawaiian/Pacific Islander, Unknown, or Other.
[d] Cattle density was not known for multiple counties with high case counts

Table 2.2 Demographic, epidemiologic, and clinical characteristics associated with urban versus rural residence.

Characteristics	Urban residents (n=119) No. (%)	Rural residents (n=73) No. (%)	OR (95% CI) [b]	*p* value [c]
Age (years)				
0-9	46 (83.6)	9 (16.4)	9.3 (3.69, 23.56)	<0.0001
10-18	14 (77.8)	4 (22.2)	-	0.003
19-40	17 (35.4)	31 (64.6)	1.0	-
41-65	34 (60.7)	22 (39.3)	2.8 (1.27, 6.26)	0.01
≥65	8 (53.3)	7 (46.7)	2.1 (0.62, 6.97)	0.22
Sex				
Male	62 (62.0)	38 (38.0)	1.0	-
Female	54 (60.7)	35 (39.3)	0.9 (0.53, 1.70)	0.85
Self-reported race [d]				
White/Caucasian	82 (78.1)	54 (81.8)	1.0	-
Non-White/Other	23 (21.9)	12 (18.2)	1.3 (0.58, 2.75)	0.70
Hispanic ethnicity				
No	84 (63.2)	49 (36.8)	-	-
Yes	8 (61.4)	5 (38.5)	-	1.0
Arab ethnicity				
No	60 (57.1)	45 (42.9)	-	-
Yes	24 (96.0)	1 (4.0)	-	0.0001
Any travel in the past month				
No	59 (67.1)	29 (33.0)	1.0	0.006
Yes	27 (44.3)	34 (55.7)	0.4 (0.20, 0.77)	
Type of travel in the past month				
None	59 (67.1)	29 (33.0)	1.0	-
Domestic	14 (42.4)	19 (57.6)	0.4 (0.20, 0.80)	0.01
International	14 (51.9)	13 (48.2)	0.5 (0.22, 1.27)	0.15
Drinking water				
Municipal, bottled	73 (61.3)	46 (38.7)	1.0	-
Any well water	12 (44.4)	15 (55.6)	0.5 (0.22, 1.17)	0.11
Exposure to livestock				

Table 2.2 (cont'd)

No	83 (60.6)	54 (39.4)	-	-
Yes	3 (23.1)	10 (76.9)	-	0.02
Hospitalized				
No	77 (56.6)	59 (43.4)	1.0	-
Yes	34 (73.9)	12 (26.1)	2.2 (1.04, 4.55)	0.04

[a] Number of isolates may not add up to the total for some variables due to missing data. For each category, percentages were calculated using the number with each characteristic as the denominator

[b] 95% confidence interval for the odds ratio (OR). ORs were calculated for urban residence relative to rural residence.

[c] The Fisher's Exact test was used for variables with fewer than 5 in one cell; no ORs could be calculated

[d] Self-reported race categories in the online Michigan Disease Surveillance System questionnaire were Caucasian, African American, Asian, American Indian/Alaska Native, Hawaiian/Pacific Islander, Unknown, or Other.

Table 2.3 Univariate and multivariate analysis identifying predictors of hospitalization among 214 patients with *Campylobacter jejuni* infections.

Characteristics	No. of Cases[a]	Hospitalized No.	(%)	OR (95% CI) [b]	*p* value [c]
Demographics					
Age (years)					
0-9	50	6	(12.0)	-	1.0
10-18	17	5	(29.4)	-	0.12
19-40	44	5	(11.4)	1.0	-
41-65	56	23	(41.1)	-	0.001
≥65	15	7	(46.7)	-	0.007
Sex					
Male	95	24	(25.3)	1.0	-
Female	84	22	(26.2)	1.0 (0.54, 2.01)	0.89
Self-reported race					
White/Caucasian	130	29	(22.3)	1.0	0.01
Non-White/Other [d]	34	15	(44.1)	2.7 (1.24, 6.08)	
Hispanic ethnicity					
No	131	41	(31.3)	-	0.18
Yes	12	1	(8.3)	-	
Arab ethnicity					
No	104	39	(37.5)	-	0.007
Yes	23	2	(8.7)	-	
Residence by county					
Rural	71	12	(16.9)	1.0	-
Urban	111	34	(30.6)	2.2 (1.04, 4.55)	0.04
Exposure history					
Season infected					
Winter, Spring	49	9	(18.4)	1.0	-
Summer, Fall	133	37	(27.8)	1.7 (0.76, 3.88)	0.19
Any travel in the past month					
No	87	32	(36.8)	1.0	0.007
Yes	61	10	(16.4)	0.3 (0.15, 0.75)	
Travel in the past month					
None	87	32	(36.8)	1.0	-
Domestic travel only	33	6	(18.2)	0.4 (0.14, 1.02)	0.05

Table 2.3 (cont'd)

International travel only	27	4	(14.8)	-	0.03
Drinking water					
Municipal, bottled	118	36	(30.5)	-	0.25
Any well water	27	5	(18.5)	-	
Clinical symptoms					
Diarrhea with blood					
No	108	33	(30.6)	1.0	-
Yes	63	13	(20.6)	0.6 (0.28, 1.23)	0.16
Abdominal pain					
No	50	16	(32.0)	1.0	-
Yes	123	30	(24.4)	0.7 (0.33, 1.41)	0.30
Body aches					
No	122	30	(24.6)	1.0	-
Yes	51	16	(31.4)	1.4 (0.68, 2.88)	0.36
Fatigue					
No	99	21	(21.2)	1.0	-
Yes	72	25	(34.7)	2.0 (1.00, 3.91)	0.05
Nausea					
No	99	18	(18.2)	1.0	-
Yes	73	28	(38.4)	2.8 (1.40, 5.61)	0.003

Multivariate analysis [e]	**Adjusted OR (95% CI)**	***p* value**
Age 41 to 65 years	6.1 (2.37, 15.70)	0.003
Age ≥65 years	10.5 (2.63, 42.19)	0.003
Non-White/Other race [d]	4.8 (1.62, 14.01)	0.009
International travel	0.3 (0.07, 0.94)	0.04
Nausea	2.8 (1.15, 6.68)	0.03

[a] Number of isolates may not add up to the total for some variables due to missing data.
[b] 95% confidence interval for the odds ratio (OR)
[c] The Fisher's Exact Test was used for variables with fewer than 5 in one cell; no ORs could be calculated
[d] Other (Non-White) race includes the following self-reported categories: Asian, Black/African American, and Other.
[e] Multivariate results were generated using forward stepwise logistic regression while controlling for variables with p-values ≤0.2 in the univariate analysis as well as potential confounders. The variables were: age, sex, residence location (urban versus rural),

Table 2.3 (cont'd)

season (fall and summer versus spring and winter), race (Non-White versus White/Caucasian), international travel, domestic travel, and symptoms (fatigue and nausea). The Homer and Lemeshow Goodness-of-Fit test indicates that the model is supported (Chi-
Square=5.36; degrees of freedom=7; p=0.62) despite using only 133 of the 214 case records. Adjusted ORs were calculated, and the Wald Chi-Square test was used to determine significance with 95% Wald Confidence Limits

Table 2.4 Univariate analysis to identify factors associated with any ciprofloxacin resistance (CIP) and any tetracycline resistance (TET) among 214 *Campylobacter jejuni* isolates from Michigan, 2011-2014.

Characteristics [a]	Any CIP resistance (n=49)			Any TET resistance (n=120)		
	No. (%)	OR (95% CI) [b]	*p* value [c]	No. (%)	OR (95% CI) [b]	*p* value [c]
Age (years)						
0-9 (n=65)	14 (21.5)	0.7 (0.31, 1.65)	0.43	39 (60.0)	1.1 (0.53, 2.32)	0.77
10-18 (n=18)	2 (11.1)	-	0.21	6 (33.3)	0.4 (0.12, 1.14)	0.08
19-40 (n=54)	15 (27.8)	1.0	-	31 (57.4)	1.0	-
41-65 (n=59)	16 (27.1)	1.0 (0.42, 2.21)	0.94	33 (55.9)	0.9 (0.45, 1.98)	0.87
≥65 (n=17)	2 (11.8)	-	0.21	10 (58.8)	1.1 (0.35, 3.20)	0.92
Sex						
Male (n=110)	23 (20.9)	1.0	-	58 (52.7)	1.0	-
Female (n=96)	23 (24.0)	0.8 (0.44, 1.62)	0.60	54 (56.3)	1.2 (0.66, 2.00)	0.61
Self-reported race [d]						
White/Caucasian (n=137)	32 (23.4)	1.0	-	76 (36.5)	1.0	-
Non-White/Other (n=35)	9 (25.7)	1.1 (0.48, 2.67)	0.77	17 (48.6)	0.8 (0.36, 1.59)	0.46
Arab ethnicity						
No (n=106)	27 (25.5)	-	-	53 (50.0)	1.0	-
Yes (n=25)	3 (12.0)	-	0.19	17 (68.0)	2.1 (0.84, 5.35)	0.10
Season						
Winter, Spring (n=56)	12 (21.4)	1.0	-	38 (67.9)	1.0	-
Summer, Fall (n=158)	37 (23.4)	1.1 (0.54, 2.34)	0.76	82 (51.9)	0.5 (0.27, 0.97)	0.04
Any travel in the past month						
No (n=88)	13 (14.8)	1.0	-	45 (51.1)	1.0	-
Yes (n=61)	21 (34.4)	3.0 (1.37, 6.68)	0.005	37 (60.7)	1.5 (0.76, 2.86)	0.25

Table 2.4 (cont'd)

Type of travel in the past month						
None (n=88)	13 (14.8)	1.0	-	45 (51.1)	1.0	-
Domestic (n=33)	4 (12.5)	-	1.0	18 (56.3)	1.1 (0.51, 2.56)	0.74
International (n=27)	17 (63.0)	9.8 (3.69, 26.09)	<0.0001	18 (69.2)	2.2 (0.85, 5.46)	0.10
Type of drinking water						
Municipal, bottled (n=119)	25 (21.1)	1.0	-	60 (50.4)	1.0	-
Any well water (n=27)	5 (18.5)	0.9 (0.29, 2.48)	0.77	19 (70.4)	2.3 (0.95, 5.75)	0.06
Poultry consumption						
No (n=16)	4 (25.0)	-	-	10 (62.5)	1.0	
Yes (n=115)	24 (20.9)	-	0.75	61 (53.0)	0.7 (0.23, 1.99)	0.48
Any animal contact						
No (n=54)	12 (22.2)	1.0	-	30 (55.6)	1.0	-
Yes (n=96)	20 (20.8)	0.9 (0.41, 2.07)	0.84	52 (54.2)	0.9 (0. 48, 1.85)	0.87
Contact with livestock						
No (n=137)	31 (22.6)	-	-	71 (51.8)	-	-
Yes (n=13)	1 (7.7)	-	0.30	11 (84.6)	-	0.04
Cattle density in resident county [e]						
Low <8400 cattle (n=23)	3 (13.0)	-	-	12 (52.2)	1.0	-
High ≥8400 cattle (n=82)	21 (25.6)	-	0.27	50 (61.0)	1.4 (0.56, 3.63)	0.45
Residence type						
Rural (n=73)	18 (24.7)	1.0	-	45 (61.6)	1.0	-
Urban (n=119)	25 (21.0)	0.8 (0.41, 1.62)	0.56	62 (52.1)	0.7 (0.37, 1.22)	0.20
Hospitalized						

Table 2.4 (cont'd)

No (n=136)	27 (19.9)	1.0		80 (58.8)	1.0	-
Yes (n=46)	14 (30.4)	1.8 (0.83, 3.76)	0.14	21 (45.7)	0.6 (0.30, 1.15)	0.12

[a] Not all numbers add up to the total number of cases per category due to missing data for some variables or the exclusion of susceptible isolates.
[b] The 95% confidence interval (CI) for the odds ratio (OR) is presented; ORs were calculated separately for CIP and TET relative to all other isolates.
[c] The Fisher's Exact Test was used for variables with ≤5 in one cell; no ORs could be calculated
[d] Self-reported race categories in the online Michigan Disease Surveillance System questionnaire were Caucasian, African American, Asian, American Indian/Alaska Native, Hawaiian/Pacific Islander, Unknown, or Other.
[e] Cattle density was not known for multiple counties with high case counts.

Table 2.5 Epidemiological factors associated with any ciprofloxacin resistance (CIP) versus only tetracycline resistance (TET) among 135 patients with resistant infections.

Characteristics [a]	Any CIP resistance (n=49) No. (%)	Only TET resistance (n=83) No. (%)	OR (95% CI) [b]	*p* value [c]
Age (years)				
0-40 (n=83)	31 (37.4)	52 (62.5)	1.0	-
≥41 (n=48)	18 (37.5)	30 (62.5)	1.0 (0.48, 2.10)	0.99
Sex				
Male (n=65)	23 (35.4)	42 (64.6)	1.0	-
Female (n=59)	23 (39.0)	36 (61.0)	1.2 (0.41, 1.78)	0.85
Residence type				
Rural (n=48)	18 (37.5)	30 (37.5)	1.0	-
Urban (n=70)	25 (35.7)	45 (64.3)	0.9 (0.43, 1.98)	0.84
Season				
Winter, spring (n=39)	12 (30.8)	27 (69.2)	1.0	-
Summer, fall (n=93)	37 (39.8)	56 (60.2)	1.5 (0.67, 3.30)	0.33
Any travel in the past month				
No (n=49)	13 (26.5)	36 (73.5)	1.0	-
Yes (n=41)	21 (51.2)	20 (48.8)	2.9 (1.20, 7.02)	0.02
Type of travel in the past month				
None (n=49)	13 (26.5)	36 (73.5)	1.0	-
Domestic (n=19)	4 (21.1)	15 (79.0)	-	0.76
International (n=21)	17 (81.0)	4 (19.1)	-	<0.0001
Type of drinking water				

Table 2.5 (cont'd)

Municipal, bottled (n=67)	25 (37.3)	42 (62.7)	1.0	-
Any well water (n=20)	5 (25.0)	15 (75.0)	-	0.42
Poultry consumption				
No (n=16)	4 (40.0)	6 (60.0)	-	-
Yes (n=115)	24 (35.3)	44 (64.7)	-	0.77
Contact with livestock				
No (n=79)	31 (39.2)	48 (60.8)	-	-
Yes (n=11)	1 (9.1)	10 (90.9)	-	0.09
Hospitalized				
No (n=85)	27 (31.8)	58 (68.2)	1.0	-
Yes (n=26)	14 (53.9)	12 (46.2)	2.5 (1.02, 6.14)	0.04

Multivariate analysis [d]	**Adjusted OR (95% CI)**	***p* value**
Age	1.0 (0.98, 1.02)	0.87
Female	0.5 (0.17, 1.40)	0.18
Urban residence	1.0 (0.33, 2.83)	0.95
Summer or fall infection	3.7 (1.03, 13.47)	0.04
International travel only	14.9 (4.00, 55.57)	<0.0001
Hospitalized	3.0 (0.78, 11.19)	0.11
Well water	0.6 (0.16, 2.26)	0.44
Livestock contact	0.2 (0.02, 2.25)	.21

[a] Number of isolates may not add up to the total for some variables due to missing data; percentages were calculated using the number with each characteristic as the denominator

[b] 95% confidence interval for the odds ratio (OR). ORs were calculated for ciprofloxacin resistance relative to tetracycline resistance.

Table 2.5 (cont'd)

[c] The Fisher's Exact test was used for variables with fewer than 5 in one cell; no ORs could be calculated
[d] Multivariate results were generated using forward stepwise logistic regression while controlling for variables with p-values ≤0.2 in the univariate analysis as well as potential confounders. A base model consisted of the following variables: age (continuous), female sex, urban residence, season (fall and summer), and international travel. Each additional variable was added separately to the base model. The Homer and Lemeshow Goodness-of-Fit test (p>0.05) was examined to ensure support for each model. Adjusted ORs were calculated and the Wald Chi-Square test was used to determine significance with 95% Wald Confidence Limits.

Figure 2.1 Percentage of the 214 *Campylobacter jejuni* isolates with **A)** resistance to eight different antibiotics and **B)** distinct antibiotic resistance phenotypes. The multidrug resistant (MDR) phenotype includes isolates with resistance to CIPAZIERYCLITEL, CIPTETCLI, and CIPTETTEL. All isolates with CIP resistance were also resistant to nalidixic acid. Abbreviations: AZI, azithromycin; CIP, ciprofloxacin; CLI, clarithromycin; ERY, erythromycin; FFN, phenicol; GEN, gentamicin; TEL, telithromycin; TET, tetracycline.

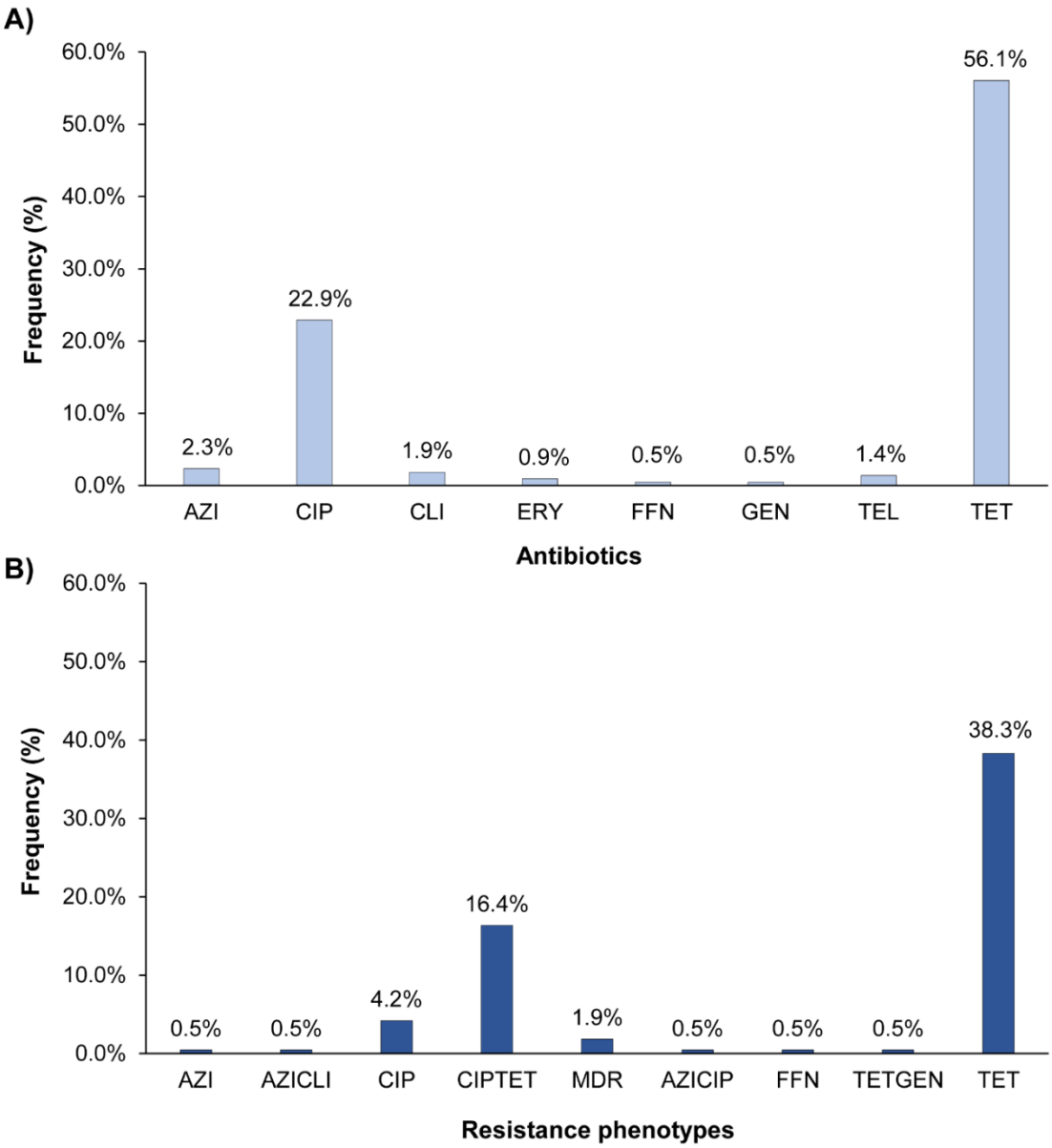


Figure 2.2 Changes in the frequency of the most common antibiotic resistance phenotypes among 214 *Campylobacter jejuni* isolates over a four-year period in Michigan. Multidrug resistant (MDR) isolates were resistant to CIPAZIERYCLITEL, CIPTETCLI, and CIPTETTEL. Abbreviations: CIP, ciprofloxacin; TET, tetracycline; AZI, azithromycin; ERY, erythromycin; CLI, clindamycin; TEL, telithromycin.

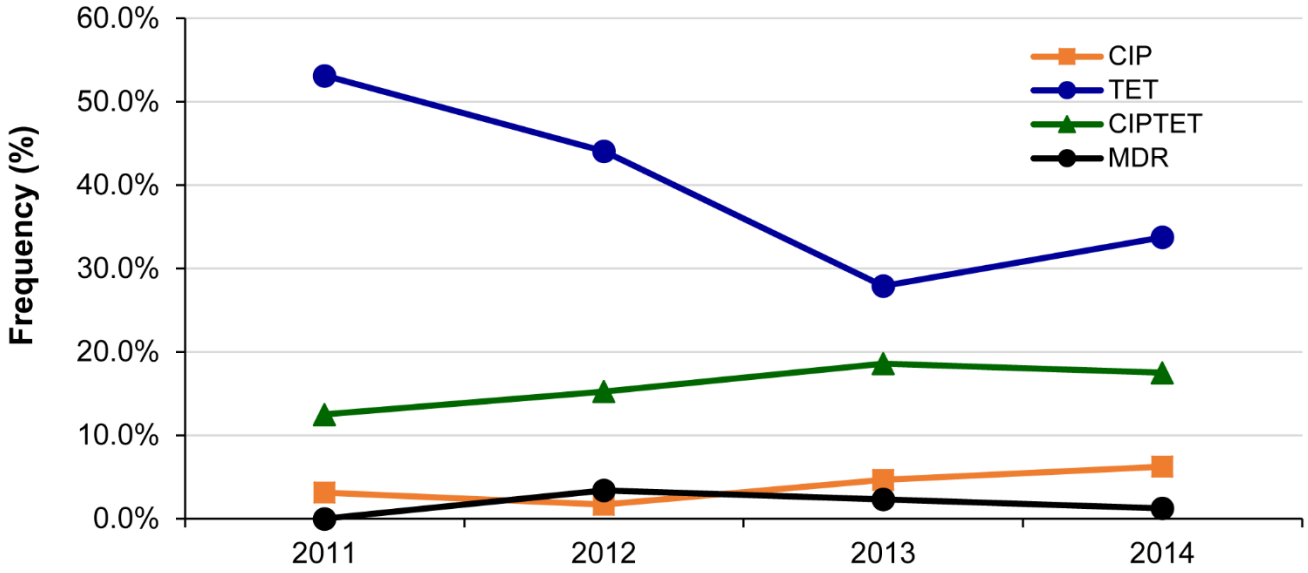
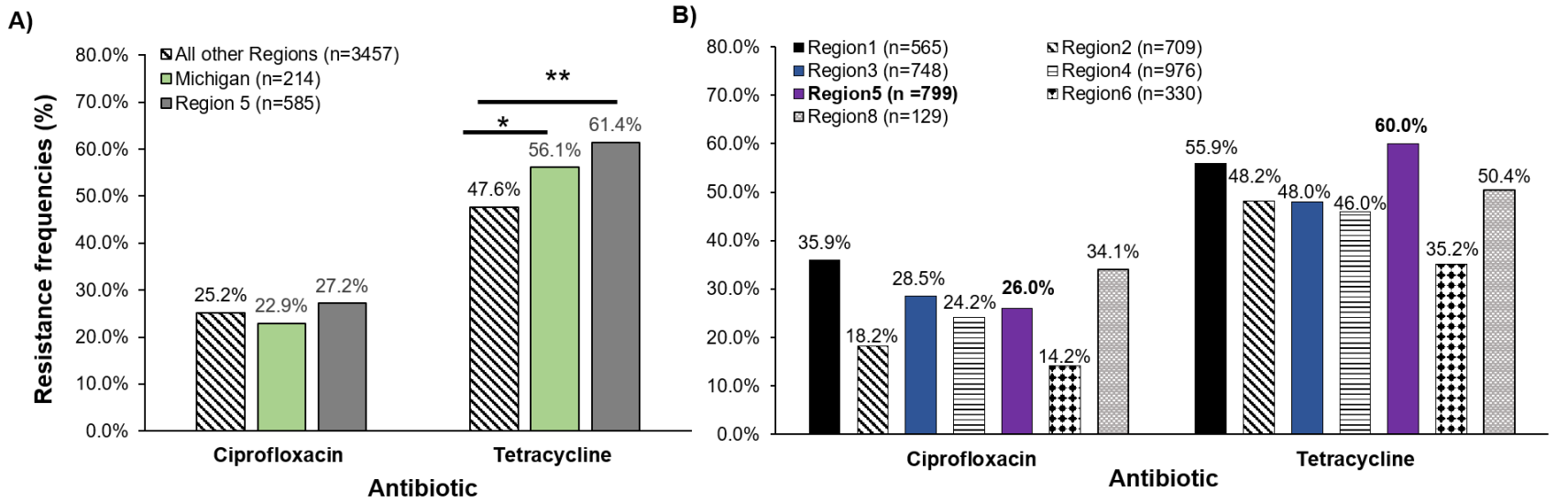


Figure 2.3 Antibiotic resistance frequencies of *Campylobacter jejuni* strains recovered from four Michigan hospitals (n=214) in 2011-2014 as compared to the National Antimicrobial Resistance Monitoring System (NARMS) data for the same time period. **A**) Michigan frequencies were compared to NARMS data from Region 5, which included Minnesota (representing Ohio, Indiana, Michigan, Illinois, Wisconsin, and Minnesota plus 34 federally recognized tribes), and the total national data (excluding Region 5). **B**) Michigan frequencies were added to Region 5 national data (n = 585) leaving a total of 799 strains in the Midwest region for comparison to NARMS regions 1, 2, 3, 4, 6, and 8. * $p \leq 0.05$, ** $p \leq 0.0001$; χ^2 test. The 10 FoodNet sites representing Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, Tennessee, California, Colorado, and New York send data captured by the state public health laboratories to NARMS to represent the different regions. Data from Region 1 (Connecticut, Maine, Massachusetts, New Hampshire, Rhode Island, and Vermont), Region 2 (New Jersey, New York, Puerto Rico, and the Virgin Islands), Region 3 (Delaware, District of Columbia, Maryland, Pennsylvania, Virginia, and West Virginia), Region 4 (Alabama, Florida, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, and Tennessee), Region 6 (Arkansas, Louisiana, New Mexico, Oklahoma, and Texas), and Region 8 (Colorado, Montana, North Dakota, South Dakota, Utah and Wyoming) were included in the analysis. Regions 7, 9, and 10 did not have data available for *C. jejuni* from 2011 – 2014 for comparison.



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CHAPTER 3: PANGENOMIC DIVERSITY, POPULATION STRUCTURE AND GENOMIC EPIDEMIOLOGY OF *CAMPYLOBACTER JEJUNI* FROM PATIENTS WITH GASTROENTERITIS IN MICHIGAN

ABSTRACT

Campylobacter jejuni is one of the leading causes of foodborne disease in the United States (US), and antibiotic resistant *C. jejuni* has been characterized as a serious public health threat. Although multilocus sequence typing is useful for differentiating *C. jejuni* strains for molecular epidemiological studies, it provides a less comprehensive view of bacterial characteristics than whole-genome sequencing (WGS). Consequently, we used WGS to examine the pangenome and investigate the genomic epidemiology of *C. jejuni* strains recovered from patients with gastroenteritis in Michigan between 2011-2014. Among the 214 strains evaluated, 83 unique multilocus sequence types (STs) were identified and 135 (63.1%) were resistant to at least one antibiotic. Core-gene phylogenetic reconstruction identified three clades, with Clade I comprising six subclades (IA-IF) and predominating (83.2%) among the strains. The core-gene phylogeny was recapitulated by a neighbor-net phylogeny showing that recombination plays a critical role in defining the population structure of *C. jejuni* (pairwise homoplasy index p=<0.01). Assessment of horizontally acquired genes identified *C. jejuni* as a reservoir for antibiotic resistance genes (ARGs); 59.8% (n = 128) of the 214 strains had at least one horizontally acquired gene. In all, 12 unique ARGs were detected representing the β-lactam, tetracycline and aminoglycoside antibiotic classes. A high level of concordance was observed between the antibiotic resistance phenotypes and ARG profiles. To examine strains circulating in Michigan, those recovered from patients reporting no recent travel were examined separately and compared to those reporting travel. Notably, travel was a significant contributor to the pangenomic diversity and ST diversity of the *C. jejuni* strains. Further stratification by urban and rural residence identified some STs such as ST-922,

to be unique to rural counties. Moreover, phylo-temporal analyses identified variation in the Clade and subclade distributions. Strains representing subclade ID, for example, increased significantly over the four-year period (CMH; *p* value = 0.023) and were more likely to have ciprofloxacin resistance than all other strains (OR 2.3, 95% CI; 1.2-4.5). Together, these data suggest that widespread geographical mixing, recombination, and horizontal gene transfer play critical roles in shaping the population structure of *C. jejuni.* In addition, the use of more comprehensive genomic typing methods demonstrates that travel or *C. jejuni* acquisition outside of Michigan enhances the genomic diversity of the strain population. Similarly, differences in the proportion and type of strains circulating in rural versus urban areas suggest that risk factors and exposures vary by residence. It is also important to note that these factors or areas are likely selecting for strains with specific traits such as those representing subclade ID with resistance to ciprofloxacin that have been increasing in frequency. These findings highlight the importance of WGS and pan-genomic analyses for enhancing our understanding of specific pathogen populations and their distributions, dissemination, and evolution as well as guiding the development of public health prevention and mitigation strategies for *C. jejuni*.

INTRODUCTION

Campylobacter spp*.* are gram-negative pathogens that have been attributed to the greatest incidence of gastroenteritis (campylobacteriosis) worldwide (1, 2). In the United States (US), *C. jejuni* represents the most common cause of bacterial foodborne illness (3) contributing to 13,000 hospitalizations and 120 deaths in the US annually (4). The Centers for Disease Control and Prevention (CDC) monitors *C. jejuni* and other foodborne

pathogens through an active surveillance system, the Foodborne Disease Surveillance Network (FoodNet), which has estimated that 14 cases of *C. jejuni* gastroenteritis are diagnosed per 100,000 individuals each year (2, 5). The most common presentation of campylobacteriosis is self-limiting gastroenteritis lasting 7-10 days with diarrhea, cramping, abdominal pain and vomiting as the predominant symptoms (2).

Fluoroquinolone (FQ) and macrolides are first line antibiotics used to treat campylobacteriosis (6), however, resistance to these antibiotics has been increasing in frequency throughout the US (7). Consequently, the CDC has classified drug-resistant *Campylobacter* spp. as "serious" public health threat contributing to 448,400 antibiotic resistant infections each year (8). To continuously examine antibiotic resistance trends and threats across the FoodNet sites in the US, the National Antimicrobial Resistance Monitoring System (NARMS) was created in 1996 (9). Although *Campylobacter* spp. have consistently been the most common foodborne pathogen reported in Michigan through the Michigan Department of Health and Human Services (MDHHS), Michigan is not a FoodNet site (10). Hence, data from Michigan are not counted in regional or national estimates that include data from only the 10 FoodNet sites representing 5-10% of the US population (11). Importantly, we have previously observed differences in antibiotic resistance frequencies over time in Michigan and relative to frequencies reported at the regional, state, and national level via NARMS for both *C. jejuni* (see Chapter 2; (12)) and non-O157 Shiga toxin-producing *Escherichia coli* (13). This geographic variation in resistance frequencies amongst enteric pathogens suggests the importance of differential selective pressures, circulating strain populations, or varying exposures and risk factors.

An assessment of circulating *C. jejuni* strains using multilocus sequencing typing (MLST), which was shown to be a useful typing method in epidemiologic and ecologic studies (14–20), identified specific sequence types (STs) and clonal complexes (CCs) to be correlated with antibiotic resistance phenotypes. For example, strains belonging to ST-982 were more likely to have tetracycline resistance and represented the predominant lineage in a subset of clinical isolates from Michigan recovered in 2011-2012 (21). More recently, the use of whole-genome sequencing (WGS) has become more widespread in public health surveillance systems. The CDC converted from molecular typing methods such as pulsed-field gel electrophoresis via PulseNet, to WGS methods in 2019 (22–24). While WGS can be used to probe antibiotic resistance frequencies by detecting resistance determinants and more rapidly detecting outbreaks, it is not often used by public health agencies to investigate broader questions in genomic epidemiology and pathogen evolution.

Relative to MLST, several WGS studies have redefined the population structure of *Campylobacter* spp. (17, 25–40). Specific lineages, for instance, have been identified in cattle and chicken, important reservoirs of pathogenic *Campylobacter* spp. (2). These specific lineages represent specialists that are often restricted to at least one host (33). By contrast, generalist lineages have a broader distribution and are typically recovered from multiple environments and hosts including wild birds (31, 33, 41, 42). CC-21 and CC-45, for example, have been identified in multiple studies amongst different hosts and environments and are distributed globally (33, 42, 43). Chicken- and cattle-specialist lineages such as ST-353 and ST-982, respectively, also have a widespread distribution, although varying frequencies have been reported in Europe, the United Kingdom, South

America, and the US (31, 32, 44, 45). The differential abundance of lineages across geographic regions may reflect the conditions that enable bacterial survival and expansion in the respective niche along with unique risk factors and exposures that influence *Campylobacter* acquisition and subsequent infection. (31, 36–38). Using WGS to define the distribution of lineages and strain characteristics in specific geographic locations is important for informing outbreak investigations and epidemiological studies. It can also aid in the development of unique programs, policies, and public health interventions to reduce the number of human infections in an area. Moreover, WGS can provide information regarding the distribution of important genes such as antibiotic or virulence genes and help identify discriminatory gene clusters within the *C. jejuni* and *Campylobacter* pangenome. Identifying specific pathways that may contribute to pathogenesis can also be performed (29), further highlighting the usefulness of WGS for the study of foodborne pathogens. Herein, we utilized WGS to comprehensively examine 214 clinical *C. jejuni* strains recovered from patients with campylobacteriosis identified via active surveillance in Michigan from 2011 to 2014.

MATERIALS AND METHODS

Bacterial strains

As described previously (Chapter 2), 217 *C. jejuni* isolates were recovered from patients in Michigan collected via active surveillance at four hospitals in collaboration with the MDHHS (12). Among these isolates, 214 were recovered from Michigan residents and three were from patients who resided in Georgia, New Jersey, and Ohio but were diagnosed with campylobacteriosis while visiting Michigan. These three out-of-state

isolates were excluded from downstream analyses that were restricted to Michigan residents. Prior to sequencing, isolates were grown at 37^{o}C on Tryptone Soy Agar (TSA) supplemented with 5% sheep blood in microaerophilic conditions as described previously (Chapter 2; (12, 46)).

Whole-genome sequencing (WGS) and bioinformatics

Bacterial DNA was isolated using the Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA) as described (12, 21, 47). Libraries were prepped with the Nextera XT library prep kit (Illumina, San Diego, CA, USA) for sequencing on the MiSeq platform (Illumina) with 2x250 bp reads at the Michigan State University Research Technology Support Facility (RTSF) or the Michigan Department of Agriculture and Rural Development.

WGS analysis methods were adapted from those described for Shiga toxin-producing *Escherichia coli* (48, 49). Briefly, raw reads were processed by trimming the adaptor sequences using Trimmomatic v.0.36 (50) with a four-base sliding window, a minimum PHRED score of 15, and a minimum length of 35. Quality control was performed using FastQC v4.10.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) to assess read quality and confirm the absence of adapter sequences. *De novo* genome assembly was performed using Spades v3.15.2 (51) (kmers 21, 33, 55, 77, 99, 127) with error correction to minimize mismatching. QUAST (52) was used with reference genome NC_002163.1 available through the National Center for Biotechnology Information (NCBI), while MultiQC v1.10.1 (53) was used to assess genome quality. Prokka v1.14.6 allowed for the annotation of each genome with “usegenus” and parameters available for

genus *Campylobacter* (54). Genomes were filtered by the number of contigs, assembly size, GC content, and the number of predicted coding genes.

Among the 217 sequenced strains, three were excluded due to poor quality, leaving an assembled set of 214 contiguous sequences for analysis. Only the 211 strains recovered from Michigan residents were included in the final analyses. A subset of 87 strains from cases without history of recent travel outside of Michigan were also selected to represent the population of strains specific to Michigan.

Pangenome and phylogenetic evolutionary reconstructions

The Roary pangenome pipeline (55) was used with -i (blastp of 95%) and the -e parameter (PRANK (56) aligner 170427) to create a multiFASTA alignment of core genes. The sequence of each core gene was concatenated into a single alignment, which was used to generate a maximum likelihood phylogeny with RAxML (57). Four gamma categories for rate heterogeneity were used with the parameters -m GTRGAMMA -N and 100 bootstrap replicates; the phylogeny was visualized using the Interactive tree of life (IToL) (55). “Roary plots.py” (https://github.com/sanger-pathogens/Roary/tree/master/contrib/roary_plots) was used to generate data plots containing metadata as well as the gene presence and absence matrix. A neighbor-net tree based on the core gene alignment was also generated using SplitsTree v4.17.0 (58) and the pairwise homoplasy index (PHI) was calculated to detect significant evidence for recombination between genomes (59). Finally, the Microbe Genome Atlas pipeline (60) was utilized to determine the average nucleotide identity (ANI). This pipeline utilizes FastANI (61) to estimate the

ANI and generate a pairwise assessment of all orthologous genes across the queried strains.

In situ molecular typing and phenotypic testing

Antimicrobial resistance phenotypes were previously determined by standard microdilution test with the Sensititre System (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Cleveland OH, US) (See Chapter 2, (12)). Contigs were queried for resistance genes and seven commonly used MLST loci to classify into STs and CCs using the ABRicate and StarAMR programs, which interface with the PubMLST database (62). These programs also use curated databases, Resfinder 4.0 (63) and PointFinder (64), to identify genes and point mutations previously shown to be important for antibiotic resistance.

Data analysis

CC and ST host specialism, or specialist lineages, were defined based on prior reports that linked specific lineages with certain sources including cattle, chickens, and humans (25, 29, 33, 35, 41, 42). Demographic, exposure, and clinical data were retrieved from the Michigan Disease Surveillance System (MDSS) and managed using Microsoft Excel and SAS Enterprise Guide 8.3. Univariate analyses were conducted to assess relationships between clades identified in the pangenome analysis and antibiotic resistance phenotypes. Odds ratios (OR) and their 95% confidence intervals (CIs) were calculated to describe the magnitude of each association, while the Fisher's exact test was used for variables with small sample sizes representing less than five per cell. A *p*

value ≤ 0.05 was considered significant for each test. The county of residence was used to determine the longitude and latitude of the public health departments located within each county to map cases by residence; MicroReact was used for the phylogeographic and temporal analyses as described (65). SAS, Epi Info™ 7, and R packages, ape (66) and ggtree (67), were used for all statistical analyses. Bioinformatic scripts can be found at Github [https://github.com/RodriguesJA]

RESULTS

Genomic characteristics of *C. jejuni* recovered from Michigan patients

Among the 214 *C. jejuni* strains with adequate sequencing data, the number of contigs ranged from 3-332 per genome with a median of 85 (**Supplementary Table 1**). The N_{50} values ranged between 3,000 and 332,500 base-pair sequences (bps), while the assembly size ranged from 1,431,867 bps to 1,889,890 bps with an average GC content of 30.5%. The genome completeness, a measure of the fraction of the genome that is assembled as compared to a reference genome (52, 68), ranged from 76.0% to 98.1% with a median of 93.2% and average of 92.4%.

An analysis of the seven MLST loci sequences from the 214 genomes identified 83 unique STs that varied in frequency (**Figure 3.1A**). Five new STs with unique allele combinations were identified. One fourth of the STs (25.7%) were represented by only one strain (singleton), whereas 11 STs comprised five or more isolates each. Overall, ST-353 (n=17, 7.9%), ST-982 (n=16, 7.5%), and ST-50 (n=11, 5.1%) predominated making up 20.6% (n=44) of the entire strain set. The 83 STs grouped together within 19 predefined CCs (**Figure 3.1B**). CC-21, which has been described as a “super-complex” comprising STs 21, 48 and 206 from multiple environments (69), was the most prevalent

(n=70, 32.7%). Another 16.8% (n=36) of the strains belonged to CC-353, while CCs 257 (n=12; 5.6%), 464 (n=8; 3.7%), 607 (n=7; 3.3%), and 283 (n=3; 1.4%) were also detected.

Based on the ST and CC designations, many CCs were also classified according to previously described host sources (33, 42). Specialist lineages were defined as those that were predominantly identified in a specific host, whereas generalists represented those lineages that have been isolated from multiple environments and hosts. Among the 214 strains examined, the chicken specialist lineages comprised 43.5% (n=93) of the strains and included ST-45 within CC-45 (n=13; 6.1%) as well as ST-50 within CC-21 (n=11; 5.1%) among others (**Figure 3.1C**). While several cattle-associated CCs were detected (n=34; 15.9%), the overall number was less than the number of chicken-associated lineages. The cattle specific lineages included: CC-61 (n = 5; 2.3%), ST-806 within CC-21 (n=3; 1.4%), CC-403 (n=2; 1.0%), CC-42 (n=2; 1.0%), CC-22 (n=2; 1.0%), and two STs within CC-21, ST-982 (n=16; 7.5%) and ST-8 (n=4; 1.9%).

By contrast, generalist lineages that were previously associated with more than one host and/or multiple environments comprised 20.1% (n=43) of all strains (**Figure 3.1C**). CC-48 (n=14; 6.5%) was the most prevalent followed by CCs 21 (n=12; 5.6%), 206 (n=9; 4.2%), and 52 (n=7; 3.3%); only one isolate belonged to CC-45. The remaining 44 (20.6%) strains, however, were either not assigned to a CC (n=13) or belonged to a CC that was not previously associated with a specific host or source.

Pangenome analysis and identification of core genes

Using Roary (55, 70), an alignment of the 214 assembled and annotated genomes was generated resulting in 8,781 unique gene loci (**Figure 3.2**). The phylogeny highlights

the diversity of this pathogen population as most strains can be found on separate branches within multiple clusters. In the heat map, a diverse array of gene clusters was observed across strains with descendant and distant lineages showing evidence for gene loss and gain. Notably, a distinct cluster representing approximately 10 strains located in the middle of the phylogeny shows that multiple genes are missing.

Among all 8,781 unique genes, 615 were categorized as core genes due to their presence in ≥ 99% or at least 211 of strains. These core genes represent only 7.0% of the total pangenome. A subset of 357 genes was classified as soft-core genes, which are defined as being present in 95%-99% of all genomes (**Figure 3.3**). When combined with the 615 core genes, these soft-core genes comprised 11.1% of the *C. jejuni* pangenome. In all, the largest group of genes was defined as accessory genes comprising 88.9% of the pangenome, with 1,169 loci representing the shell genes. These shell genes are classified based on their presence in 15%-95% of strains, while the remaining 6,640 loci were classified as cloud genes present in <15% of strains.

An examination of the number of protein-coding genes per genome also revealed substantial diversity between strains ranging from 1,387 to 2,008. Most of these genes were strain-specific as 2,699 singleton genes belonged to only one strain, comprising 30.7% of the pangenome (**Figure 3.4**). An analysis of the average nucleotide identity (ANI) values across genomes demonstrated a range from 96.7 % to 99.9 % with an average of 98.4% and median value of 98.5%.

Core-gene alignment of *C. jejuni* strains resolves three clades

Construction of a maximum likelihood phylogenetic tree based on 550,736 bp representing the 615 core genes revealed three unique clades with >80% bootstrap support (**Figure 3.5**). The phylogeny shows a diverse population of *C. jejuni* with evidence of evolutionary separation. Some strains in each of the three clades were highly similar and grouped together with up to 100% bootstrap support. Other strains, however, were less similar and located on distinct branches of the phylogeny with less than 70% bootstrap support. Among the three clades, Clade I was the largest and most diverse representing 68 STs that could be further divided into six related subclades, IA – IF, with a range of 94.0% to 100.0% bootstrap support. Several strains within all three clades were highly related and found on the same branch.

Most of the *C. jejuni* strains belonged to Clade I (n=178; 83.2%) followed by Clades II and III, which represented 31 (14.5%) and 5 (2.3%) strains, respectively (**Figure 3.6**). The distribution of the Clade I subclades, IA-IF, also varied among the 214 strains. Overall, subclade IF predominated (n=78; 43.8%) out of the 178 Clade I strains, yet subclade ID also contained a high percentage of strains (n=70; 39.3%). Fewer strains were classified as subclades IA, IB, IC, and IE and ranged from 5 to 10 strains per group.

Distribution of STs and antibiotic resistance profiles across the phylogeny

Within the core gene phylogeny, most STs were clustered together, although some were located on different branches (**Figure 3.5**). For example, 63.6% (n=7) of the 11 strains belonging to ST-45 were clustered together within Clade II with 100% bootstrap

support. However, within the larger subclades, ID and IF, several of the STs were located on different branches.

The antibiotic resistance phenotypes also varied in their distribution across the core gene phylogeny. Multidrug resistance (MDR), defined as phenotypic resistance to three or more antibiotic classes, was only identified in four strains. Notably, two MDR strains with resistance to all macrolide, macrolide-like, and streptogramin (MLK) antibiotics plus ciprofloxacin, grouped together (100% bootstrap support) within Clade II despite belonging to different STs. Another MDR strain with MLK, tetracycline and ciprofloxacin resistance belonged to subclade ID of Clade 1 and another with resistance to clindamycin, ciprofloxacin, and tetracycline clustered within subclade IF. Two additional azithromycin resistant strains grouped separately within Clades II and III, while another strain with azithromycin plus clindamycin resistance belonged to subclade IE on a different part of the phylogeny. The one strain with phenicol resistance grouped within Clade II and shared the same ST (508) as one of the azithromycin resistant strains.

Although the strains with ciprofloxacin resistance were found throughout the phylogeny, ciprofloxacin resistant strains were twice as common within subclade ID as compared to all other strains (OR: 2.3; 95% CI: 1.2-4.5). Indeed, 32.9% (n=23) of strains within Clade ID (n=70) were resistant to ciprofloxacin (**Table 3.1**). By contrast, tetracycline resistance was not concentrated within one clade. For example, subclade IB (n=10), which contained strains representing ST-922, were all resistant to tetracycline (Fisher's Exact Test; *p* value=0.005), while tetracycline resistance was significantly less likely to occur in Clade II strains relative to all others (OR: 0.4; 95% CI: 0.20-0.96).

Recombination is evident within *C. jejuni* genomes

Evidence of recombination between *C. jejuni* strains was demonstrated within an unrooted phylogeny based on the alignment of 615 core genes (**Figure 3.8**). Similar to the rooted core gene phylogeny (**Figure 3.5**), the predefined STs spanned multiple branches of the phylogeny. For instance, the 13 STs belonging to CC-353 were found in three separate clusters, while strains belonging to CCs 257, 353, 52 and 48 were each found in two different clusters within the phylogeny–and across subclades IC-IF. Moreover, strains belonging to STs 572 and 2782 were differentially placed on the phylogeny among multiple strains representing CC-48 as opposed to the pre-assigned CCs 206 and 21.

To further assess if recombination played a role in shaping the *C. jejuni* population structure within Michigan, a neighbor-net phylogeny was also constructed using the 615 core genes (**Figure 3.9**). Not surprisingly, there was significant evidence for recombination (PHI; p value <0.01) across the 214 genomes. This network also resolved the three clades that were identified by the RaXML maximum likelihood phylogeny based on the same 615 core genes (**Figure 3.5**). A uniquely bifurcating tree, however, was produced rather than central clades. As compared to the maximum likelihood phylogeny, subclade IB was separated into two unique groups. One group includes strains belonging to ST-467 within CC-49, while the other was distinct representing ST-922 that lacked a pre-assigned CC. The neighbor-net phylogeny demonstrated phylogenetic reticulations and intermingling between the generalist and specialist lineages. For example, subclade ID contained strains from chicken associated lineages CC-607, CC-464, CC-354, CC-353, as well as a few strains from the generalist CC-52 lineage. The most predominant

subclade, IF, comprised both generalist and specialist lineages mixed within the larger super-complex containing ST-21. Cattle specialist lineages were separated into four distinct clusters, the largest of which were isolates from ST-982 of CC-21 within subclade IF. In contrast to the maximum likelihood phylogeny, CC-61 within sublade IF and CC-21 were uniquely separated. Cattle specialists from CC-22, CC-42 and CC-403 were identified in Clades II and III, respectively.

Frequency and distribution of antibiotic resistance genes

We next examined the number of antibiotic resistance determinants among all 214 strains (**Figure 3.10**). In all, 12 unique horizontally acquired antibiotic resistance genes were detected in 59.8% (n=128) of the strains. These 12 genes are important for resistance to three large classes of antibiotics including the β-lactams, tetracyclines and aminoglycosides, although the genes for β-lactam resistance predominated. More specifically, nine different β-lactam resistance genes were identified and 149 (69.6%) of the strains contained at least one of these genes. Of these nine β-lactam resistance genes identified, *bla*$_{(OXA-193)}$ was most common and was detected in 43.9% (n=94) of the strains. None of the strains carried more than one β-lactam resistance gene. Tetracycline resistance determinants were also common as 55.6% (n=119) of the strains contained one of the two tetracycline genes identified. Among these 119 strains, 94.1% (n=112) harbored *tet*(O), while the remainder (n=7; 3.3%) had *tet*(O/32/O), a mosaic chimeric gene that was suggested to have arisen by recombination between *tet*(O) and *tet*(32) (71). Both genes encode ribosomal protection proteins that mediate tetracycline resistance (72).

Several point mutations were also detected in the 214 *C. jejuni* strains. Notably, a T86I substitution within the quinolone resistance determining region, a well-defined quinolone and fluoroquinolone resistance point mutation (73), was identified in the gyrase A gene, *gryA*, in 44 (20.6%) strains. In addition, a mutation in *rpsL* (K43R), which has been previously linked to streptomycin resistance (73), was found in one ST-9062, CC-353 strain (TW19240) representing subclade ID.

Correlation between resistance phenotypes and genotypes

To determine how well WGS pipelines correlated with antibiotic resistance phenotypes, we calculated the very major and major error as well as the sensitivity and specificity of WGS methods (**Table 3.2**). Ciprofloxacin, tetracycline, chloramphenicol, and MLK-antibiotics were assessed. The sensitivity of WGS screening for ciprofloxacin resistance was 0.90 and the specificity was 0.99. For tetracycline, the sensitivity and specificity were both 0.98. Notably, these methods did not identify mutations that confer resistance for the MLK antibiotics, while seven of strains were resistant to MLK antibiotics. Further, discrepancies were identified as resistance determinants for aminoglycoside resistance had a major error of 12.7%.

Recent travel enhances the genomic diversity of the *C. jejuni* strain population

To determine how pathogen acquisition outside of Michigan affects the diversity and population structure of *C. jejuni*, we more comprehensively examined the subset of 150 cases with a known travel history. Overall, 63 (29.9%) of the 211 Michigan residents reported traveling in the month prior to their infection, while 87 (40.7%) reported no travel.

The travel status of the remaining 64 cases was not known and therefore, they were omitted from the analysis. The three out of state cases were also excluded. Among the cases with a known travel history, 35 reported domestic travel (23.3%) and 26 (17.3%) reported international travel; two individuals failed to report the travel location. Those 87 cases with no history of recent travel defined the Michigan-specific pathogen population, which were evaluated separately for comparison to the remaining strains.

Limiting the analysis to residents reporting no travel in the past month detected differences in ANI and the pangenome analysis. First, a pairwise genomic comparison of the 87 strains calculated the ANI between orthologous gene pairs to be between 96.7% and 99.9% with a mean and median of 98.3% and 98.5%, respectively. In the pangenome analysis, strains from cases without recent travel had 6,322 genes, which is 1.4 times less than those detected among all 214 strains. Roughly 511 core and 501 soft-core genes were identified, which were 1.2 times greater and 1.4 times fewer than all 214 strains combined, respectively. In addition, the number of coding sequences (CDS) ranged from 1,387 to 1,948 with an average of 1,732 for the subset of Michigan strains, which had a comparable range (1,387-2,008) and average (1,741) CDS as all 214 strains.

Subclade IF predominated and represented 20.0% (n=30) of the Michigan strains recovered from patients without a recent travel history (**Figure 3.11A)** followed by subclade ID (n=28; 18.7%), Clade II (n=17; 11.3%), and subclade IB (n = 5; 3.3%). Only two strains were identified within subclades IE, IC and Clade III. In contrast to the 87 Michigan-specific strains, subclades IE (n=26; 17.3%) and IB (n=20; 13.3%) predominated amongst individuals who traveled followed by subclades IA (n=6; 4.0%), IF

(n=3; 2.0%) and Clade II (n=3; 2.0%). While two strains were identified within subclades ID and IC, only one Clade III strain was from a case reporting intra or international travel.

Differences were also observed after stratifying by ST (**Figure 3.11B)** and CC (**Figure 3.11C)**. In all, 41 unique STs belonging to 17 CCs were identified in the Michigan specific subset; 51.2% (n=21) of these STs were singletons. Indeed, only four STs (982, 45, 353, and 48) had greater than five strains with ST-982 representing 9.2% (n=8) of the Michigan strains. CC-21 (n=17; 19.5%), CC-353 (n=14; 16.1%), CC-45 (n=7; 8.1%) predominated in Michigan strains. The super-complex CC-21 containing CC-21, CC-48 and CC-206 comprised 31.0% (n=27) of the Michigan specific strains.

By contrast, 45 unique STs belonging to 14 different CCs were identified amongst the 63 strains from cases reporting a history of recent travel. In all, 55.6% (n=35) of the strains were classified as singleton STs with ST-353 (n=4; 6.3%) and ST-464 (n=4; 6.3%) predominating. Additionally, chicken-specialist lineages were most common among the cases reporting travel (n=27; 42.9%) as well as those reporting no travel (n=39; 44.9%) **(Figure 3.11D**). The cattle-specialist lineages, however, were more likely to be identified in the Michigan-specific subset (n=17; 19.5%) as compared to the cases reporting traveling in the past month (n=7; 11.1%).

Temporal phylogeography of *C. jejuni* in Michigan

Because the prevalence of *C. jejuni* infections has increased in frequency in Michigan over the four-year period (12), we sought to examine the phylogeographic and temporal distribution of specific lineages. An increasing frequency of infections caused by

Clade I strains was observed over time relative to Clades II and III combined (Cochran-Mantel-Haenszel (CMH); *p* value=0.03). When subclades were examined separately, subclade ID increased significantly from 23.5% (n=8) in 2011 to 41.0% (n=32) in 2014 (CMH; *p* value = 0.023) (**Figure 3.12**). There were no clear differences, however, in the distribution of clades across geographic regions based on the location of residence (https://microreact.org/project/8AcoEJBuTmeeidkjUrA6NA/e5e96c0f).

Because we previously identified different risk factors for campylobacteriosis and antibiotic resistant infections in patients from urban versus rural counties (12(12, Chapter 2), we also stratified the clades by residence type. Of the 211 cases from Michigan residents, 189 had reported county of residence and 61.9% (n=117) resided in urban counties. Not surprisingly, we identified differences based on urban versus rural residence. For example, strains representing subclades IA (n=3; 4.2%) and IB (n=6; 8.3%) were more frequently identified in rural counties (**Figure 3.13A**), which was also true within the Michigan-specific subset of strains. We also detected several STs (e.g., 922, 8, 2132, and 3510) and CCs that were only found in rural counties (**Figure 3.13, panels B and C)**. Chicken specialist lineages predominated in both urban and rural counties. Of the 72 strains identified in rural counties, 20.8% (n=15) were cattle specialists, as compared to urban counties where cattle specialist lineages only made up 11.2% (n=14) of the population (**Figure 3.13D**).

DISCUSSION

The pathogen *C. jejuni* has diverse biology that includes variation in the ability to colonize and survive in a wide range of hosts and environments and to cause differences in clinical presentation during infection. This variation was suggested to be attributable to

its genomic diversity, which enables its success as an enteric pathogen (74). Although our study is restricted to *C. jejuni* strains from Michigan patients, we have demonstrated that there is extensive genomic diversity within this pathogen population. Among the 8,781 gene loci detected, most (88.9%) of the genes belong to the accessory genome, which supports the findings of other pangenomic studies of *Campylobacter* spp. and is consistent with an open pangenome (29, 31, 32, 41). An open pangenome demonstrates an increasing total number of genes as the number of strains under study increases; hence, the number of core genes tend to decrease until the totality of the diversity in the pathogen population is represented (75). The diversity within this population was further highlighted by the number of strain-specific genes making up 30.7% of the total pangenome. This finding demonstrates the importance of evolutionary processes such as horizontal gene transfer (HGT), which involves the uptake of DNA from the environment. Indeed, *Campylobacter* are naturally competent, a biological property that promotes DNA uptake and has shaped its ability to adapt and occupy a diverse range of niches (76–79). Further evidence of the extensive genomic diversity was detected herein as ANI values ranged from 96.7% to 99.9% and ≥ 95% ANI has been detected among organisms from the same species (80). To fully classify the accessory genome, an assessment of plasmid-borne genes is needed since optimized plasmid identification tools and plasmid databases are not readily available for *Campylobacter* spp. Use of other tools and databases have identified plasmid borne genes such as *tet*(O) (81), in this strain collection, which strongly suggests the presence of the more common plasmids.

The maximum likelihood phylogeny of the 615 core gene alignment uncovered three unique clades and six-subclades within Clade I. Some STs were clustered together,

while in larger clades ID and IF, STs were located on different branches. Intriguingly, five new STs with new allele combinations were identified in different parts of the phylogeny within Clade II (n=1) and subclades ID (n=2) and IF (n=2), suggesting diversification within this strain population. Because the STs were not always grouped together within the phylogeny, it is likely that recombination further contributes to the diversification of specific lineages as well as the emergence of novel clades. Indeed, the population structure of *C. jejuni* has been described to be influenced by recombination in prior studies (32, 33).

Some antibiotic resistance phenotypes, namely ciprofloxacin and tetracycline resistance, were also distributed throughout the phylogeny, which is suggestive of co-evolutionary processes and the selection of strains with active resistance determinants for these drugs. Indeed, HGT and clonal expansion have been linked to the distribution of resistance determinants within *C. jejuni* previously (32). Additional evidence of evolutionary processes governing the population structure of *C. jejuni* were demonstrated within the unrooted phylogeny, where two strains belonging to ST-572 and ST-2782, for instance, grouped together with strains from a different CC than the original CC that was previously defined by MLST. The intermixing of strains from CCs 21, 48 and 206 provides further evidence for the classification of the super-complex ST-21 (18, 82), as these CCs were previously shown to have evidence of frequent recombination.

The constructed and annotated neighbor-net phylogeny recapitulated the previously described multilineage models that highlight the importance of recombination in *Campylobacter* (29, 33, 83). In fact, the PHI recombination test (59) was significant (p value<0.01). Nonetheless, other populations were separate, providing additional support for the previously described recombination barriers between the larger generalist

populations, CC-45 and CC-21. Recombination barriers such as ecological, mechanistic, and adaptive barriers have been suggested to shape the structure of the *Campylobacter* pangenome (83). *C. jejuni* populations isolated from a variety of environments have defined unique specialist and generalist lineages amongst the diversifying pathogen populations (33). Broad generalist lineages like CC-21 and CC-45, for instance, predominated within the Michigan strains, though chicken specialist lineages such as CC-353, and cattle specialist lineages including STs 61 and 982 within CC-21, were also identified. The identification of STs with well documented host-associations (25, 27, 29, 31, 33, 35, 38, 41, 42, 83) such as the poultry associated ST-353, highlights the importance of poultry and livestock (ST-982) (42) and other exposures as potential sources. Future work should therefore focus on characterizing strains recovered from these sources for comparison to those obtained from patients with campylobacteriosis to better understand transmission and disease risk.

Prior studies have observed differences between regional and state resistance frequencies as compared to national surveillance systems such as NARMS (See Chapter 2, (12)). Thus, identification and screening of antibiotic resistance determinants within the population enabled an assessment of clonal expansion and natural selection. Selective pressures within the environment and in different hosts could be differentially selecting for specific *C. jejuni* strains with certain traits or phenotypes. Furthermore, the distribution of host reservoirs, biogeography, and regional agricultural practices for food production, could impact the distribution and frequency of specific resistant *C. jejuni* populations in the US. Data from the resistance gene analysis identified concordance between well documented resistance genes and specific resistance phenotypes, which is consistent

with a prior study (84). However, discrepancies between some phenotypes such as resistance to MLK-antibiotics and ciprofloxacin, suggest that WGS is less useful for identifying rare or new mutations that may not be included in internationally curated databases. This finding highlights the importance of the continued use of culture-based phenotypic methods to accompany WGS for monitoring resistance frequencies and identifying emerging or re-emerging resistant pathogens along with novel resistance determinants.

Previous studies of *C. jejuni* in Michigan identified cattle as a potential reservoir for specific lineages like ST-982, and ST-61 (47, 85). These studies also identified differential risk factors between urban and rural patients with campylobacteriosis, which could be due to differing exposures or differentially distributed *C. jejuni* populations across residence types (See Chapter 2; (12, 21, 47, 85). Thus, we analyzed the pangenome of 87 strains from cases without a recent travel history to represent strains circulating in Michigan. It is notable that varying lineage frequencies were observed after stratifying by travel status, which could be related to the biogeography of the specific sub-lineage niche or host. Further stratifying by host associations that were classified based on the MLST data identified chicken specialist lineages to predominate among cases reporting recent travel (n=27; 42.9%) as well as those who did not (n=39; 44.9%). Cattle specialist lineages, however, were more likely to be identified in Michigan patients reporting no recent travel (n=17; 19.5%) than in patients reporting travel in the past month (n=7; 11.1%). Thus, exposures related to direct or indirect transmission between poultry are important for the prevention of campylobacteriosis in both travel and non-travel associated cases. Comparatively, prevention strategies for *C. jejuni* infections related to

cattle exposures may have more of an impact on disease in cases without a recent travel history. Indeed, of the 11 individuals reporting livestock exposure, 63.6% (n=7) were attributed to a cattle-associated lineage.

Stratification and phylo-temporal geographic analyses identified an increasing trend of subclade ID over time (CMH; *p* value=0.023). The composition of subclade ID, which is primarily represented by CC-353, suggests that human disease from this chicken specialist lineage increased in Michigan from 2011 - 2014. Importantly, subclade ID was also linked to ciprofloxacin resistance as compared to all other strains examined. These data highlight the importance of monitoring resistance frequencies by pathogen since clonal expansion and persistence as well as changing behaviors or risk factors may explain some of the observed trends in resistance over time. While stratification based on county did not reveal differences in the distribution of *C. jejuni* lineages, significant variation was observed when urban versus rural county was examined. Specifically, strains belonging to ST-922 in subclade IB, were found only in rural counties and ST-982 in CC-21 subclade IF predominanted in rural counties. This variation may be attributed to differences in the frequency of factors associated with campylobacteriosis in different areas in Michigan. For instance, age, ethnicity, a history of travel in the past month, livestock exposure, and consumption of well water, have been previously linked to campylobacteriosis in Michigan patients (See Chapter 2; (10, 12, 21); such factors, however, may be less important in other geographic locations.

This study represents one of a few that has explored the pangenomic diversity of *C. jejuni* strains from a specific region and is congruent with current knowledge of *C. jejuni* genomic epidemiology in the U.S. (32, 86). *C. jejuni* is a sporadic and outbreak associated

disease with several reservoirs and risk factors for disease including travel, consumption of contaminated food products and direct or indirect exposure to animal and environmental reservoirs (2). Continued monitoring of the distribution of *C. jejuni* lineages using pangenomic and host specialism data will enable the detection of emerging and re-emerging populations and could assist in epidemiologic studies to identify potential reservoirs in the absence of source data. WGS and antibiotic resistance screening are also critical for the monitoring of resistance frequencies in *C. jejuni*; however, more comprehensive databases containing the most current resistant determinants must be developed and updated. Further still, absence of phenotypic data does not allow for identification of new resistance mutations that could be critical to detect the emergence of resistant bacterial populations. Additionally, the identification of many STs, including novel, region-specific STs that are not part of a MLST-based CC, requires a more comprehensive genomic analysis to identify lineages and lineage traits that are linked to a specific reservoir host or source. Exploration of these currently undefined lineages would further support the utility of pangenomic studies to aid in our understanding of the genomic epidemiology of the *C. jejuni* strain population. Such studies could ultimately facilitate the design of new prevention strategies targeting this important cause of gastroenteritis in the U.S.

APPENDIX

Supplementary Table 1 Genomic data for the 214 patient-derived *Campylobacter jejuni* strains examined.

Table 3.1 Associations between *Campylobacter jejuni* lineages identified in the pangenome analysis based on 615 core genes and resistance to ciprofloxacin or tetracycline.

Clades[a]	Any CIP resistance (n=48)				Any TET resistance (n=120)			
	No.	(%)	OR (95% CI) [b]	*p* value [c]	No.	(%)	OR (95% CI) [b]	*p* value [c]
Clade I (n = 178)	41	(85.4%)	1.2 (0.51-3.04)	0.6	105	(87.5%)	2.0 (0.97-4.17)	0.06
Clade IA (n = 5)	0	(0.0%)	---	--	1	(20.0%)	---	0.2
Clade IB (n = 10)	0	(0.0%)	---	---	7	(70.0%)	---	0.5
Clade IC (n = 9)	1	(11.1%)	---	0.7	9	(100.0%)	----	0.005
Clade ID (n = 70)	23	(32.9%)	2.33 (1.2-4.5)	0.018	41	(58.6%)	1.2 (0.65-2.1)	0.6
Clade IE (n = 6)	1	(16.7%)	----	1.0	3	(50.0%)	---	1.0
Clade IF (n = 78)	16	(20.5%)	0.8 (0.43-1.65)	0.6	44	(56.4%)	1.0 (0.6-1.8)	1.0
Clade II (n = 31)	6	(12.6%)	0.8 (0.31-2.10)	0.7	12	(38.7%)	0.4 (0.20-0.96)	0.04
Clade III (n = 5)	1	(2.1%)	----	1.0	3	(60.0%)	---	1.0

[a] Clade I was is made up of subclades IA-IF

[b] The 95% confidence interval for the odds ratio (OR) is presented; ORs were calculated separately for any CIP and any TET relative to all other isolates. [c] The Fisher's Exact Test was used for variables with ≤5 in one cell; no ORs could be calculated

Table 3.2 Major and Minor error associated with correlation between WGS and phenotypic data. Specificity and Sensitivity of WGS screening for ARGs with antibiotic resistance phenotypes.

Antibiotic	No. with resistance phenotype	No. with resistance genotype and phenotype	Very major error £	No. susceptible phenotype	No. of susceptible genotype and phenotype	Major error€	Sensitivity	Specificity
Ciprofloxacin	48	43	5 (2.4%)	166	165	1(0.5%)	0.896	0.994
Tetracycline	120	117	3 (1.4%)	95	93	2 (0.9%)	0.975	0.979
Gentamicin	1	0	1 (0.5%)	213	186	27 (12.7%)	--	0.873
Chloramphenicol	1	0	1 (0.5%)	213	213	0	--	1
MLK Antibiotics*	6	1	5 (2.4%)	207	207	0	0.167	1

£ 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.

* MLK antibiotics include macrolides, and macrolide-like-antibiotics lincosamides and streptogramin

Figure 3.1 The distribution of *Campylobacter jejuni* **A**) sequence types (STs) and **B)** clonal complexes (CCs) as determined by extracting seven multilocus sequence typing loci from the 214 strains. **C)** The distribution of strains representing specific STs or CCs previously linked to a host reservoir (e.g., cattle- or chicken-specialists) or multiple reservoirs and environments (generalist). Those ST/CCs that have not been previously linked to a given host were classified as “unknown”.

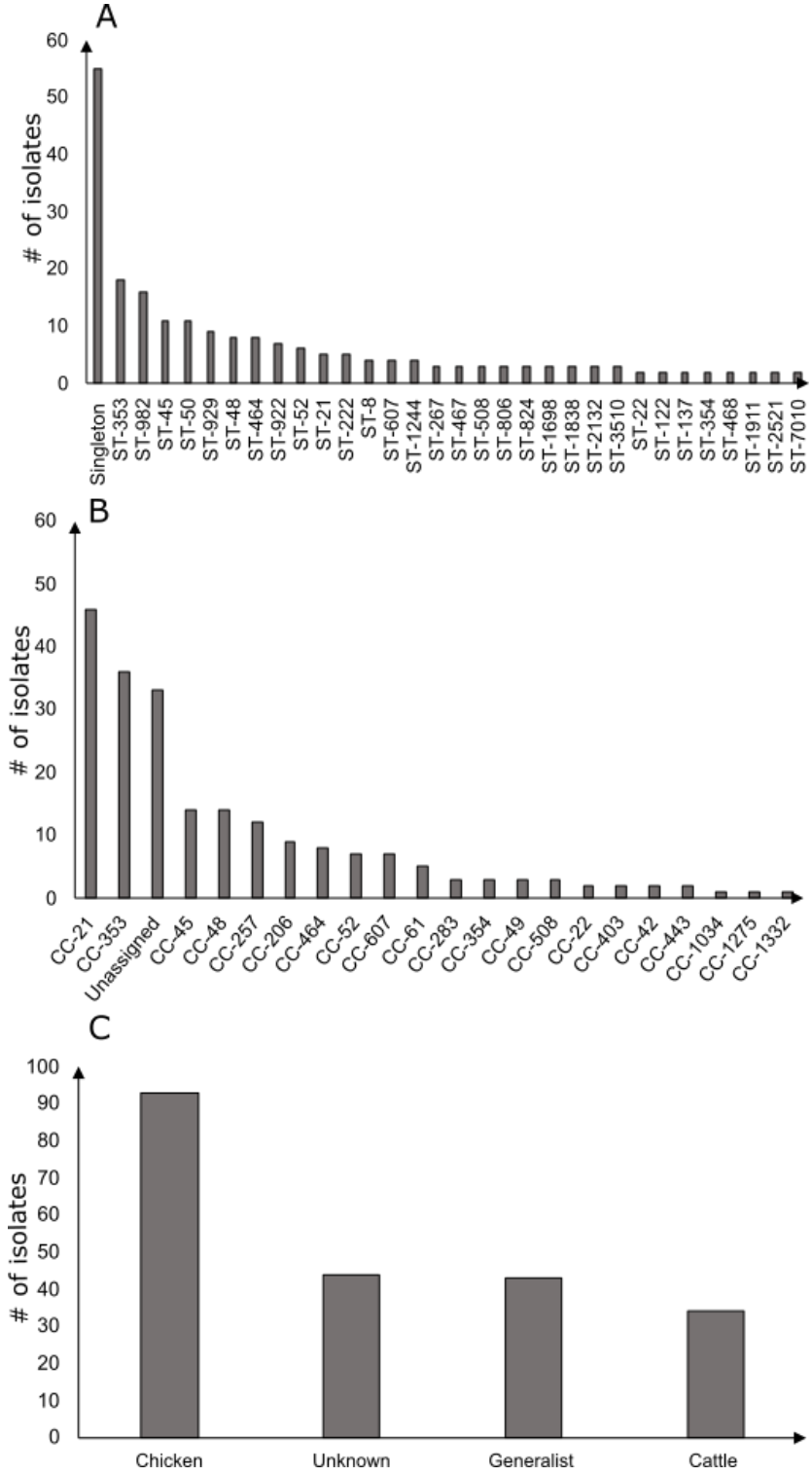

Figure 3.2 Maximum likelihood phylogeny generated by 615 core gene alignment rooted at mid-point annotated with a presence and absence of the 8781 gene clusters identified in Roary pangenomic analysis of the 214 *Campylobacter jejuni* strains. Blue shading marks the presence of a particular gene per isolate (column).

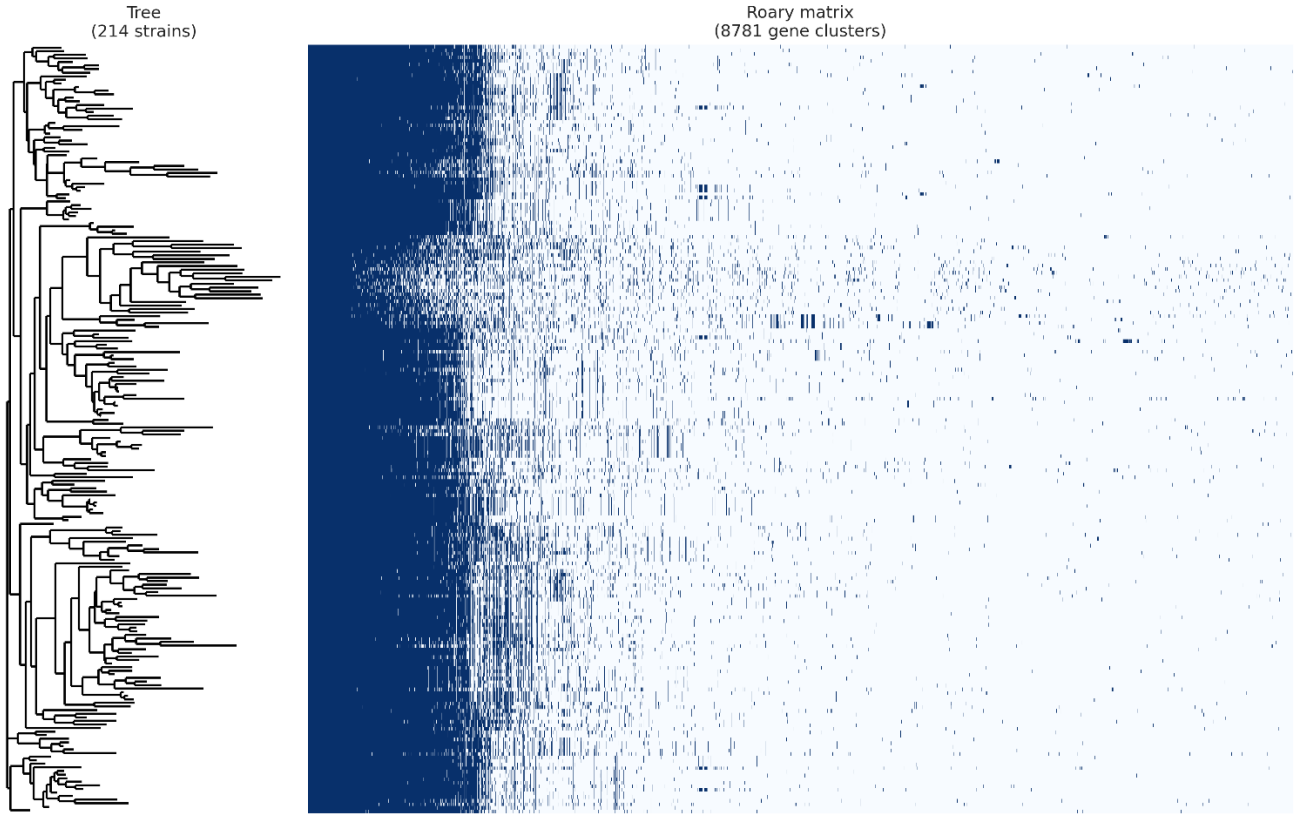

Figure 3.3 Distribution and frequency of “shared” genes identified in the pangenomic analysis of 214 *Campylobacter jejuni* strains.

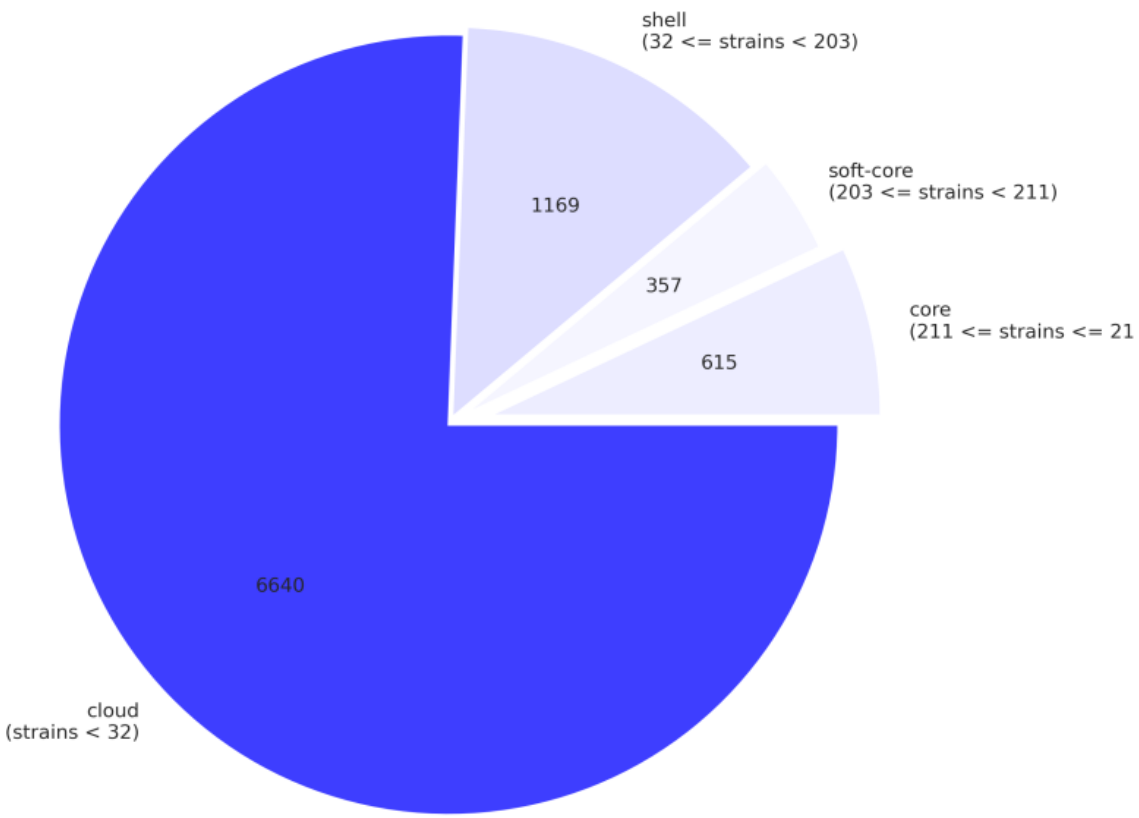

Figure 3.4 Classification and distribution of the number of genomes for each of the 8781gene clusters.

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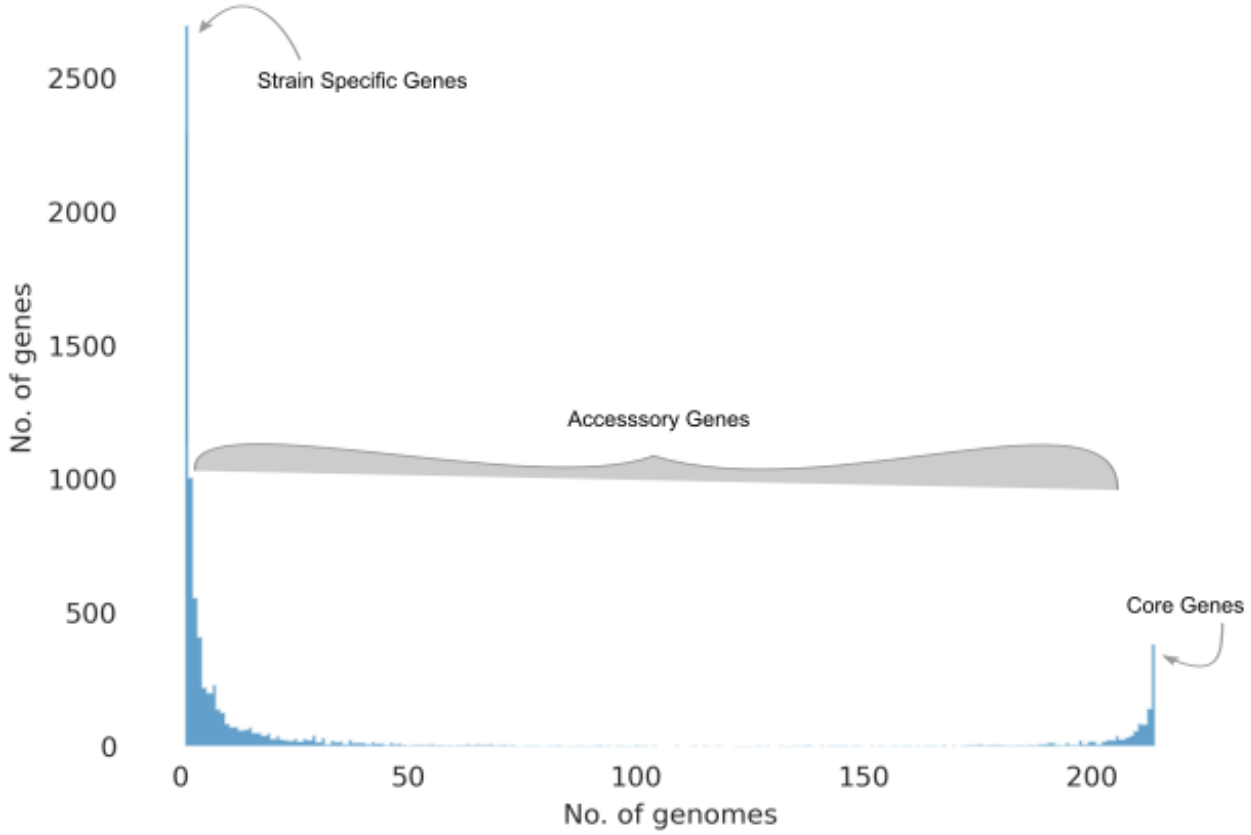

Figure 3.5 Maximum likelihood phylogeny rooted at mid-point based on the 615 core gene alignment with 100 bootstrap replicates. Clades were labeled and the subclades within Clade I are colored based on >80% bootstrap support. Sequence types (STs) are annotated per strain followed by antibiotic resistance phenotypes. Colored squares represent phenotypic resistance to the following: Phenicol, FFN; Clindamycin, CLI; Telithromycin, TEL; Erythromycin, ERY, Azithromycin, AZI; Gentamicin, GEN; Ciprofloxacin, CIP; and Tetracycline, TET.

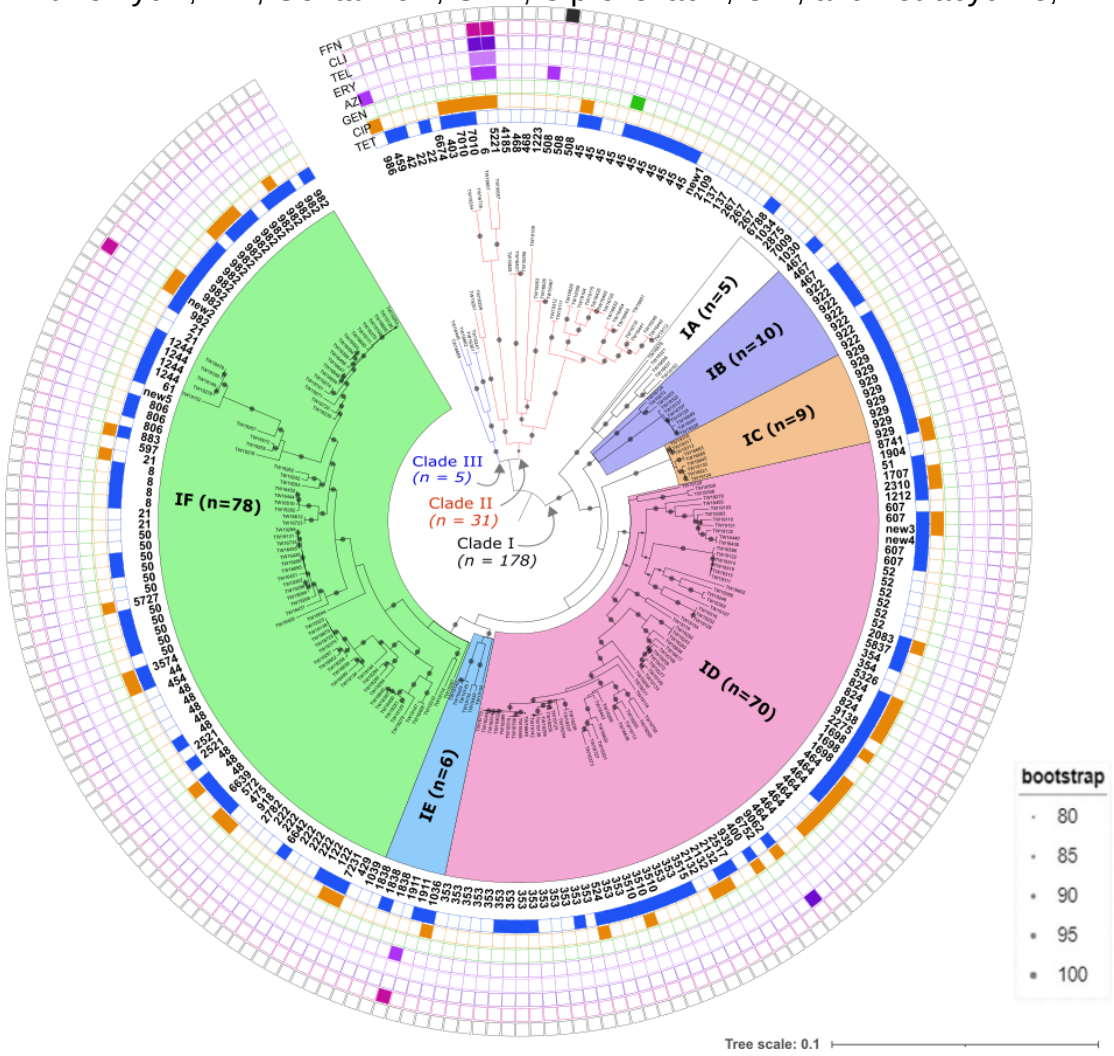

Figure 3.6 The distribution (number) of *Campylobacter jejuni* strains belonging to each clade and subclade identified by the alignment of 615 core genes.

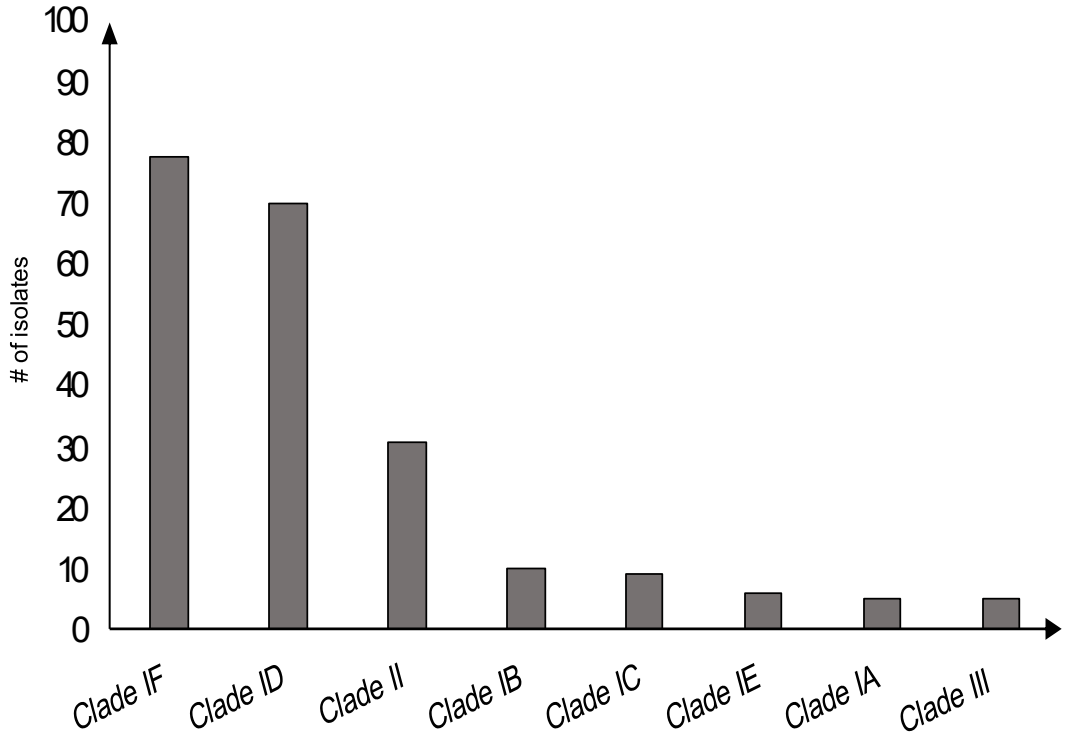

Figure 3.7 The **A**) frequency of ciprofloxacin resistance and **B**) tetracycline resistance stratified by the clade or subclade designation as determined in the pangenomic analysis.

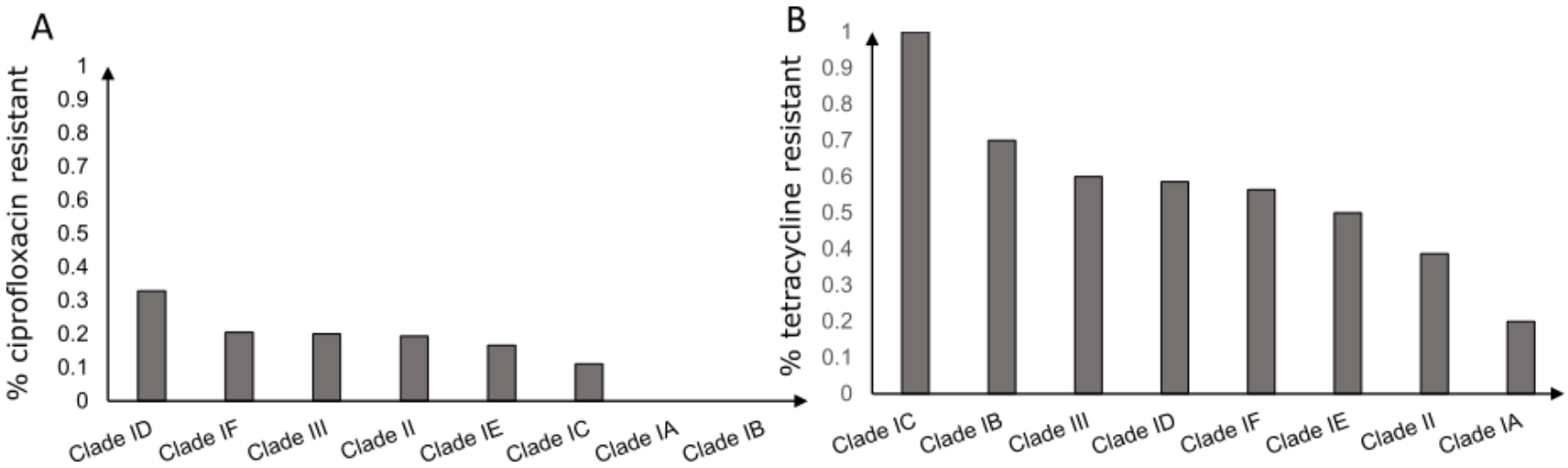

Figure 3.8 Unrooted Maximum likelihood phylogeny based on the 615 core gene alignment with 100 bootstrap replicates. Strains belonging to the same sequence type (ST) are collapsed and the nodes are proportional to the number of strains within each ST. Clades and subclades are colored, while the multilocus sequence typing (MLST)-based clonal complexes (CCs) are annotated at the outer edge. The CCs that span multiple clades are colored. STs that are differentially placed in the phylogeny relative to the preassigned MLST-based CC designation have arrows that point from STs to the pre-assigned CCs

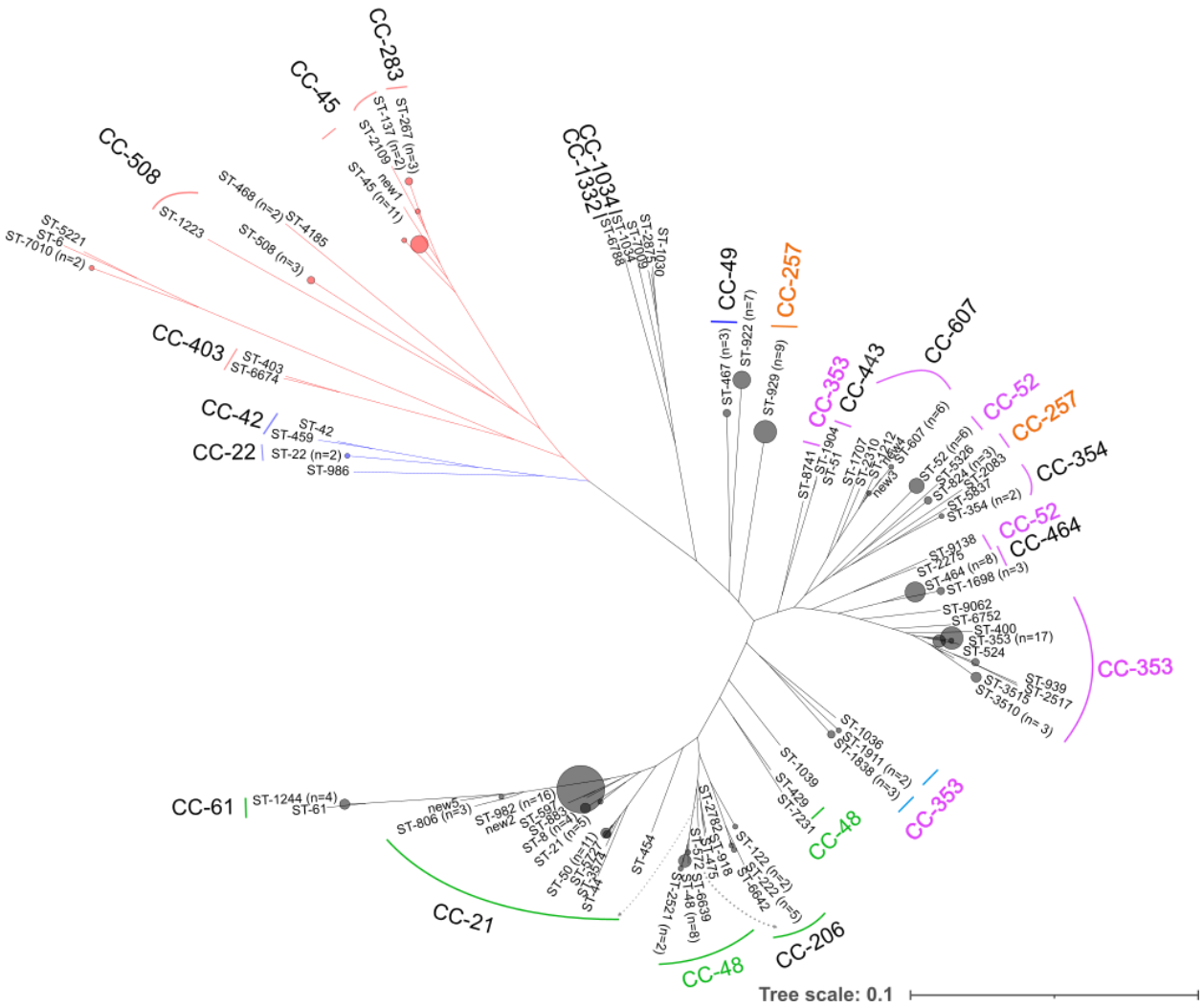

Figure 3.9 Neighbor-net phylogeny annotated with the subset of strains specific to Michigan from cases reporting no travel by host specialism category (colored dots) previously defined by multilocus sequence typing. Clades and subclades are labeled and colored with a line

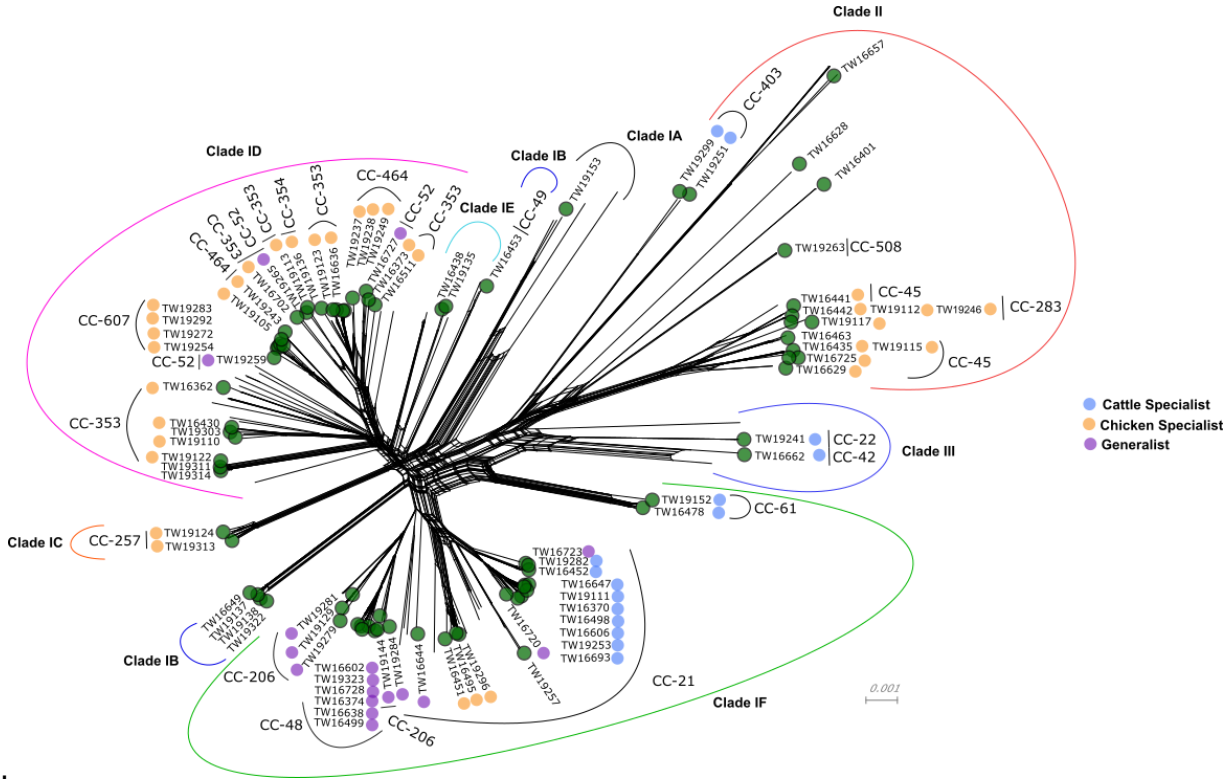

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Figure 3.10 Maximum likelihood phylogeny based on the 615 core gene alignment with 100 bootstrap replicates in 214 *Campylobacter jejuni* strains along with the presence of key antibiotic resistance genes.

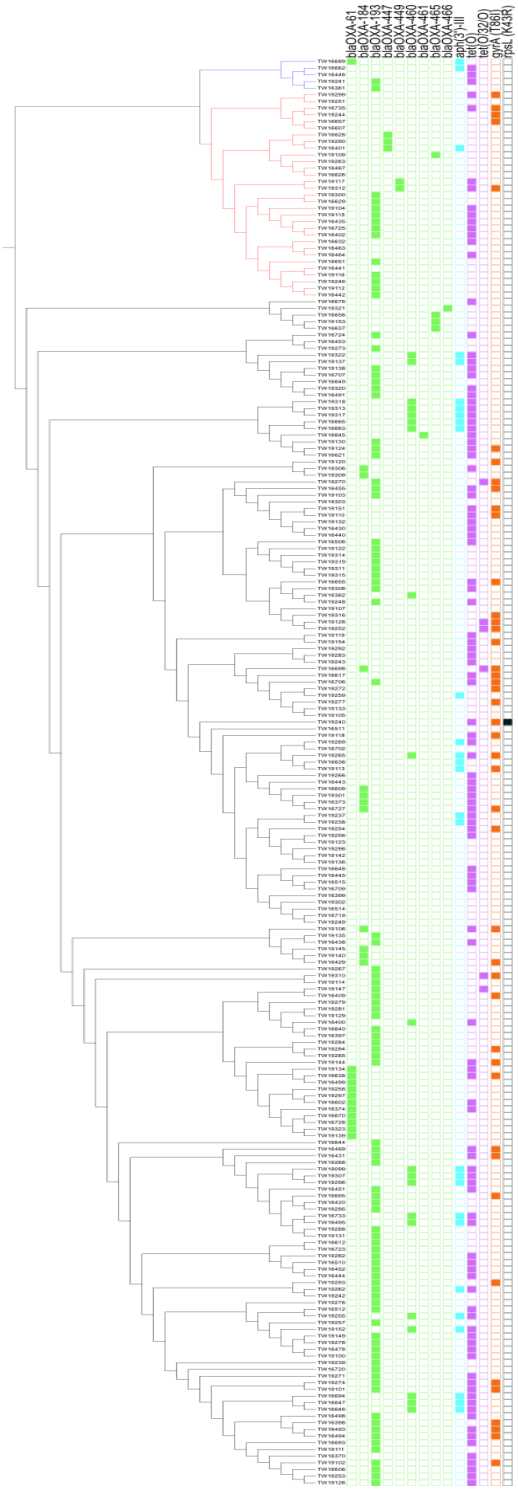


Figure 3.10 (cont'd)

Colored squares indicate the presence of a specific resistance gene within the genome; gene names are indicated at the top of each column. Antibiotic resistance genes are colored based on their functional category. Colors show different resistance categories as follows: green, beta lactams; blue, aminoglycosides; purple, tetracyclines; orange, quinolones, and fluoroquinolones; and black, MLK-antibiotics

Figure 3.11 A-D Distribution and frequency of lineages defined in the pangenomic analysis stratified by recent travel history.

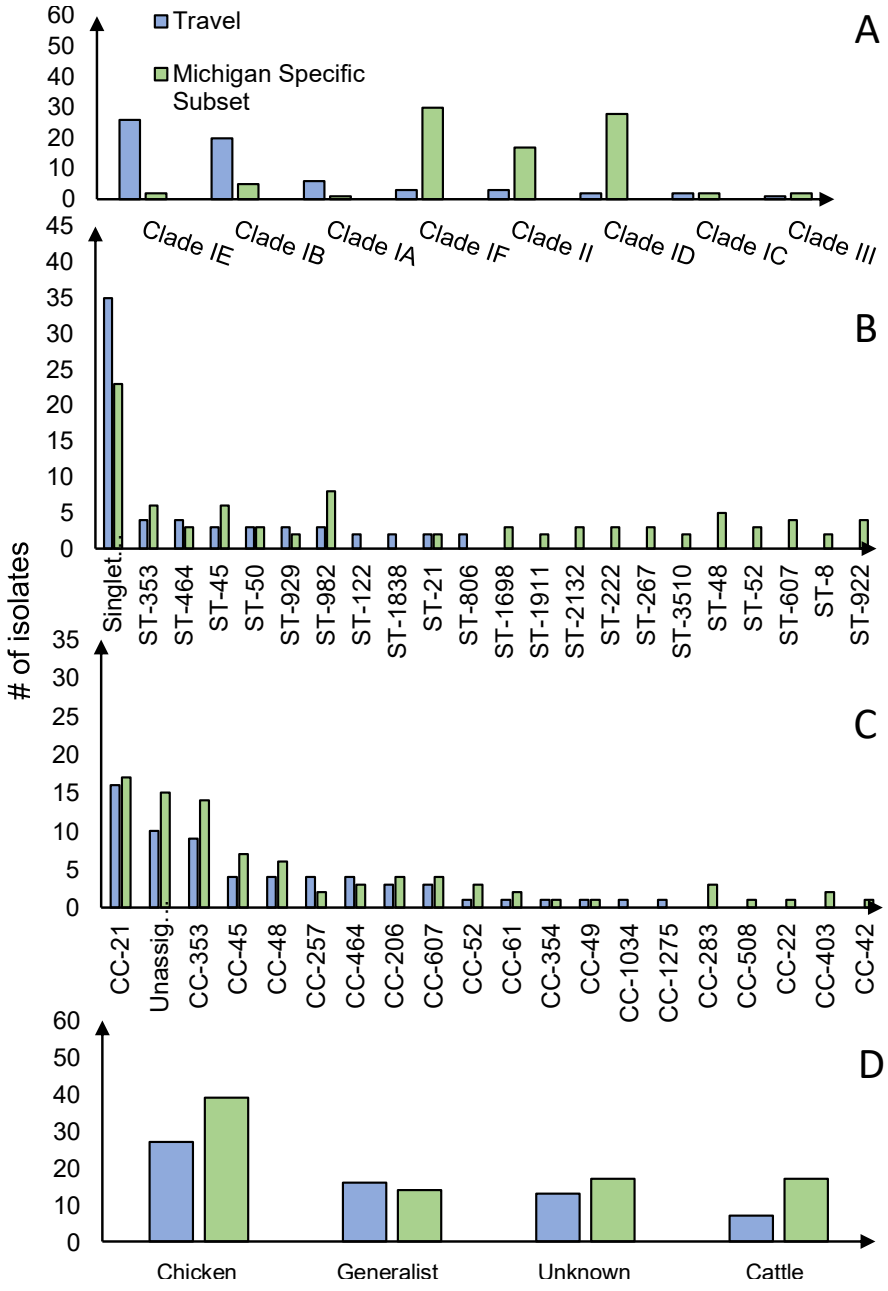
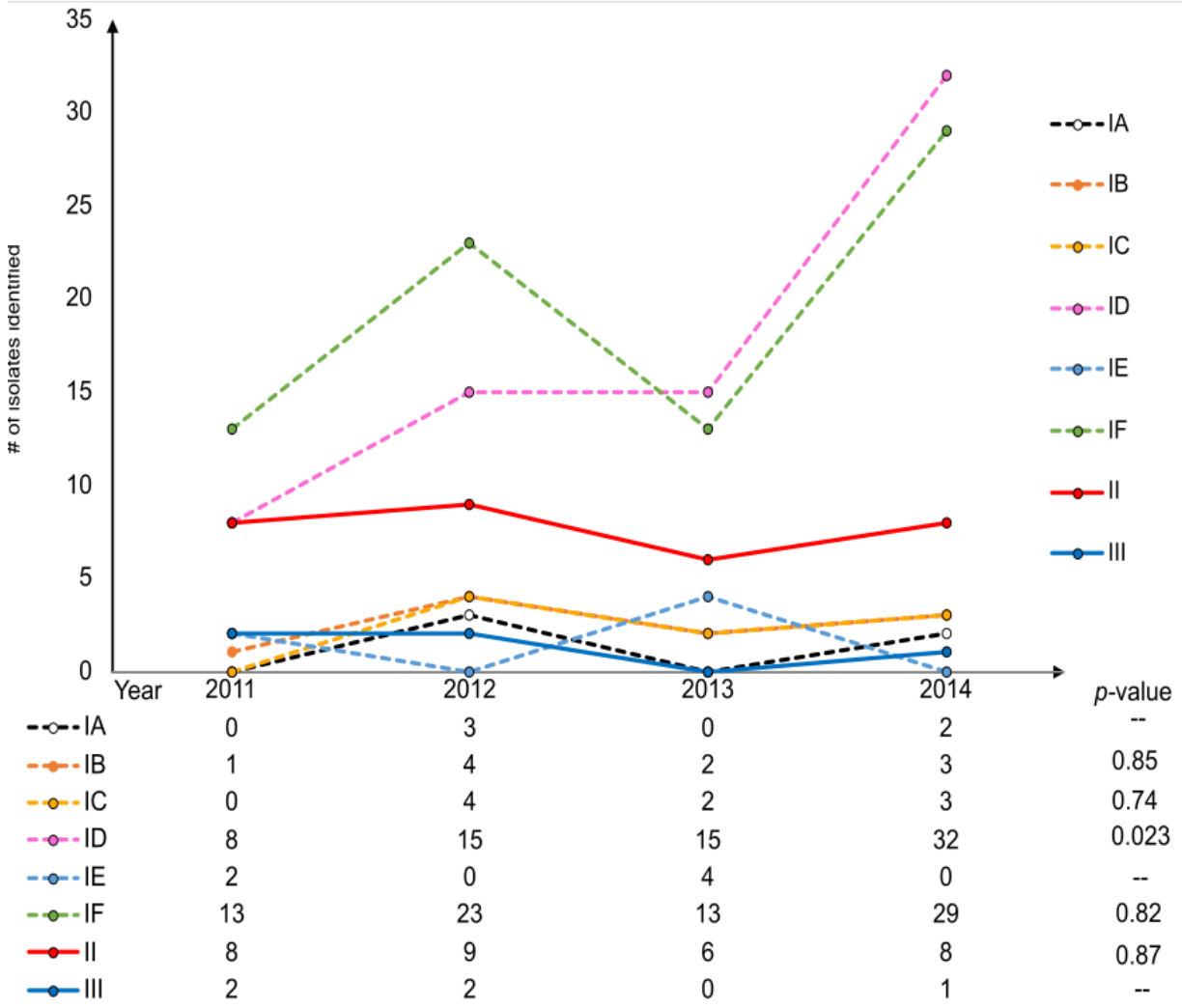
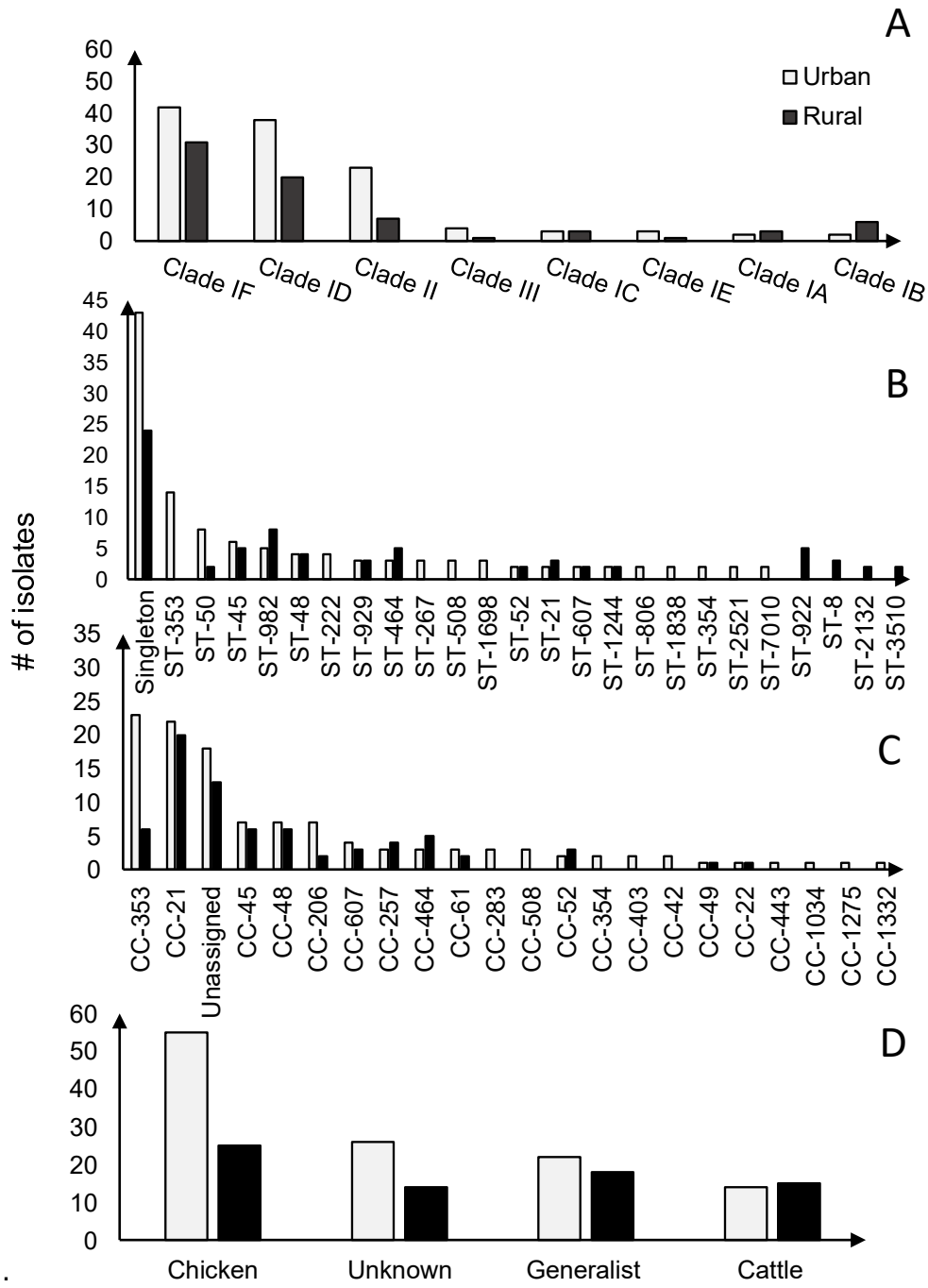


Figure 3.12 Number of *Campylobacter jejuni* clades and subclades recovered from Michigan patients between 2011 – 2014. The *p*-value was calculated using the Cochran-Mantel-Haenszel Chi-square test.



Year	2011	2012	2013	2014	*p*-value
IA	0	3	0	2	--
IB	1	4	2	3	0.85
IC	0	4	2	3	0.74
ID	8	15	15	32	0.023
IE	2	0	4	0	--
IF	13	23	13	29	0.82
II	8	9	6	8	0.87
III	2	2	0	1	--

Figure 3. 13 A-D Distribution and frequency of *Campylobacter jejuni* lineages from 214 patients stratified by urban and rural residence.



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CHAPTER 4: COMPARATIVE PHYLOGENOMIC ANALYSES REVEAL EVIDENCE OF TRANSMISSION OF HIGHLY SIMILAR *CAMPYLOBACTER JEJUNI* STRAINS BETWEEN CATTLE AND HUMANS

PREFACE

The preliminary data from the characterization of molecular epidemiologic information and characterization of antibiotic resistance of Campylobacter jejuni *isolated from cattle have been presented in this chapter has been previously published:*

Cha W, Mosci RE, Wengert SL, Vargas CV, Rust SR, Bartlett PC, Grooms DL, Manning SD. 2017. Comparing the genetic diversity and antimicrobial resistance profiles of *Campylobacter jejuni* recovered from cattle and humans. *Front Microbiol* 8:1–13.

Author Contributions Statement: WC, RM, DG, and SM conceived the study and contributed materials; RM, CV-V, SR, PB, DG, and SM planned the study and sampled the animals; WC, RM, and SW performed the experiments; WC and SM analyzed the data and drafted the paper; all authors approved the final version.

ABSTRACT

Campylobacter jejuni is the mostly commonly identified human pathogen in cases of gastroenteritis in the United States. Studies have shown that the number of *C. jejuni* outbreaks attributed to cattle through consumption of unpasteurized milk products has increased as has the frequency of antibiotic resistant infections. Previous studies examining *C. jejuni* strains from Michigan patients found specific cattle-associated multilocus sequence types (STs) such as ST-982, to predominate and more frequently have resistance to tetracycline. Tetracycline resistance frequencies were also higher in Michigan cases relative to national frequencies, suggesting that ST-982 and other cattle-

associated tetracycline resistant strains may contribute to regional variation. Hence, we used whole-genome sequencing (WGS) to more comprehensively examine ST-982 strains recovered from patients (n=15) and cattle (n=8) as well as other cattle-associated STs (n=5) to determine the likelihood of interspecies transmission. The data were used to validate a WGS clustering algorithm, average nucleotide identity (ANI) distance partition around medoids clustering (PAM) through the Microbial Genomes Atlas (MiGA), and closely related strains were further characterized using high-quality single polymorphism (hqSNP) clustering. Notably, these methods could distinguish highly related strain populations and identify clusters comprising related strains from both humans and cattle. For instance, 88 SNPs were found to separate a cattle and a human strain that were previously classified as ST-8. In contrast, human and cattle derived ST-982 strains were separated by >800 SNPs in the hqSNP analysis, however, the human strains collected in different years had only 11-36 SNP differences. These findings highlight the potential for sustained reservoirs of highly similar and distinct strains of *C. jejuni*. Such studies illustrate the usefulness of WGS analytical tools for better defining strains that may be more transmissible between species and demonstrate that both unique and diversifying pathogen populations are circulating within specific geographic locations.

INTRODUCTION

Campylobacter spp. are the most common cause of human gastroenteritis in the United States (US) and affects up to 1.3 million people annually (1). *Campylobacter* spp. have a wide host range and food animals such as poultry, cattle, and pigs, serve as

reservoirs (2). In the US, campylobacteriosis is often associated with traveler's diarrhea due to the consumption of contaminated food products or exposure to contaminated environmental and food animal reservoirs (3). Although poultry consumption has been considered the most important food-associated risk factor for campylobacteriosis (3–7), recent work has highlighted the importance of contaminated cattle products. Indeed, dairy products like unpasteurized milk have been linked to an increasing number of outbreak-associated cases (8) and source attribution studies have identified cattle as the source of 10% to 25.8% of campylobacteriosis cases (9). Moreover, campylobacteriosis cases were signifcantly more likely to report recent contact with ruminants such as cattle, while increased cattle densities were linked to an increased risk of disease (10).

Campylobacter spp. are among the most abundant human pathogens in cattle and can inhabit the gastrointestinal tract in concentrations of ~3.10^4 colony forming units (CFU)/g (11, 12). It has been estimated that the global cattle population sheds 3 x 10^{17} *Campylobacter* into the environment daily (13). Thus, there is potential for considerable spillover into the food chain and environmental reservoirs as well as transmission to other animals residing in the same agroecosystem (14–16). Monitoring these specific strain populations is therefore critically important for epidemiology and public health.

Genomic and molecular epidemiologic studies utilizing multilocus sequence typing (MLST) and more recently whole-genome sequencing (WGS), have defined specific genetic lineages to be associated with cattle. For example, strains classified as sequence type (ST)-982 as well as those that cluster within a subset of larger clonal complexes (CCs) such as CC-61, have been linked to cattle previously (17–20). More specifically, these studies identified associations between some lineages and reservoir hosts like

cattle and chickens. Strains belonging to ST-982 were also more likely to be resistant to tetracycline and associated with rural residence, cattle exposure and well water consumption (19). Cattle-associated lineages representing CC-61, for instance, have been reported in industrialized nations like the United Kingdom (18). Similarly, in Europe, ~500,000 cases of campylobacteriosis were attributed to two cattle associated lineages within, CC-61 and CC-42 (21). Previous studies in Michigan have also shown that a subset of cattle-associated lineages representing CCs 61 and 21 predominated among campylobacteriosis cases and in cattle from one beef and two dairy farms (See Chapter 3; (19, 20).

Further, our recent pangenome analysis of 615 core genes demonstrated that *C. jejuni* strains from patients clustered into three clades with the cattle-associated lineages grouping together (see Figure 5, Chapter 3). Given the close proximity of both human- and cattle-associated genomes in the core gene phylogeny, we sought to use WGS to compare the set of 214 *C. jejuni* genomes from Michigan patients examined in Chapter 3 to 72 cattle-derived genomes recovered during the same timeframe. The goal of this analysis was to assess the potential for direct or indirect transmission of *C. jejuni* between cattle and humans. Specifically, we sought to examine the population structure of *C. jejuni* based on pangenome, core genome, and high-quality single nucleotide polymorphism (hqSNP) alignments generated using multiple WGS bioinformatic pipelines. Comparing the genomic diversity and relatedness of *C. jejuni* from cattle and humans will help identify specific strain types and bacterial traits that may foster transmission. The identification of closed related strains across sources may also provide insight into emerging lineages. Combined with rigorously characterized epidemiologic data, these analyses can be used

to make real-time inferences about highly similar and dissimilar strains to inform public health action.

MATERIAL AND METHODS

Bacterial strains, DNA isolation, and antibiotic susceptibility testing

C. jejuni isolates were previously recovered via an epidemiologic study of Shiga toxin-producing *Escherichia coli* in beef and dairy cattle (22). Briefly, 220 fecal grab samples from one dairy and two beef operations in mid-Michigan were collected from July to August 2012 for *Campylobacter* culture, isolation, and antibiotic resistance phenotyping as described previously (20). Seventy-two isolates were recovered following culture on Tryptone Soy Agar (TSA) containing 5% sheep blood, cefoperzone (20 µg), amphotericin B (4 µg), and vancomycin (20 µg/mL) overnight at 37^{o}C.in microaerophilic conditions. DNA was extracted using the Qiagen DNeasy Kit (Qiagen, Valencia, CA, USA) as described (19, 20, 23), and each isolate was confirmed to be *C. jejuni* using a published PCR assay (see Chapter 2; (19, 20, 23–25). Antimicrobial resistance phenotypes were previously determined by standard microdilution test with the Sensititre System (Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Cleveland OH, US) as described in Chapter 2 and prior publications (19, 23).

Whole-genome sequencing (WGS) and bioinformatics

Previously described sequencing methods were followed (Chapter 3; (26, 27)). In brief, libraries were prepped with the Nextera XT library pre kit (Illumina, San Diego, CA, USA) and sequencing was performed on the MiSeq platform (Illumina) with 2x250 bp

reads at the Michigan State University Research Technology Support Facility (RTSF). Trimmomatic v.0.36 (28) was used to trim adaptor sequences from raw reads followed by *de novo* genome assembly using Spades v 3.15.2 (29).

FastQC v.4.10.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc), QUAST (30) and MultiQC V 1.10.1 (31) were used to assess the quality of the reads and assembled genomes. Prokka v.1.14.6 (32) was used to annotate each genome with the "usegenus" parameter available for *Campylobacter*. Through this process, 72 cattle genomes were used for pangenome and phylogenetic analyses for comparison to the 214 patient-derived genomes characterized in Chapter 3.

Pangenome and phylogenetic evolutionary reconstructions

For the comparative genome analysis, Roary (33, 34) was used to create a multi-fasta alignment of the 286 human and cattle *C. jejuni* genomes and the Microbial Genomes Atlas (MiGA) (35) was used to determine the average nucleotide identity (ANI). The MiGA pipeline utilizes FastANI (36) to estimate the ANI and generate a pairwise assessment of all orthologous genes across the queried strains. MiGA further clusters strains with the all-vs-all distance matrix using the *k*-medoid partition, or partition around medoids (PAM) (37). Thus, the MiGA pipeline can rapidly cluster related genomes based on the ANI distance matrix.

To further characterize the related bacterial populations, raw reads were input through a hqSNP analysis. LYVE-SET (38), a pipeline that utilizes a previously characterized SNP algorithm VarSCAN, and the SET algorithm to all identified hqSNPs. Next, RAXML (39) is used to construct phylogenies based on the SNP distance matrix

generated. Each hqSNP tree was generated with 100 bootstrap replicates and annotated in the Interactive Tree of Life (iTOL) v6 platform (40) with metadata including strain source, year of isolation, county of residence (human) or farm location, and resistance phenotypes reported in Chapters 2 and 3. Contigs were also queried for the seven commonly used MLST loci with the blast-based program, StarAMR, which also classified the strains into CCs and STs since it interfaces with the PubMLST database (41).

Data analysis

The host association status of specific CCs and STs, which have also been referred to as specialist lineages, was defined based on prior studies (Chapter 3; (18, 21, 42–44). Demographic, exposure, and farm metadata were retrieved and managed using Microsoft Excel and SAS Enterprise Guide 8.3.

RESULTS

Genomic characteristics of *C. jejuni* recovered from cattle in Michigan.

The N_{50} values of the 72 cattle strains ranged between 10.1 and 186.4 kilobase pairs (kbps) (**Supplementary Table 2**). Roughly 21 – 921 contigs were obtained per genome, and the assembly size ranged from 1,569,418 bps to 1,914,118 bps with an average GC content of 30.5%. The genome completeness ranged from 84.3% to 97.9% with an average of 91.1%.

Among the 72 cattle genomes, the seven MLST loci sequences were classified as 15 unique STs representing seven distinct CCs that varied in frequency (**Table 4.1**). Three strains possessed new alleles in *aspA*, *gltA, glyA*, and two new alleles were

identified in *uncA*, yielding three novel STs. In all, 36.1% (n=26) of the cattle strains belonged to ST-459, while ST-982 (n=8) and ST-1244 (n=8) both represented 11.1% of the strains. Only two strains were classified as each of the following STs: 42, 806, and 7679, and 16.7% (n=12) of the strains were unique and represented by a single ST (singletons).

The distribution of CCs was also assessed with CC-42 predominating and making up 38.9% (n=28) of the strains followed by CC-21 (n=15) and CC-61 (n=10). Not surprisingly, most (72.2%; n=52) of the strains belonged to lineages previously described as cattle specialist lineages like STs within CC-61 and CC-42. Ten (13.9%) strains, however, were classified as chicken associated lineages and four (6.9%) were generalist lineages; 8.3% (n=6) of the strains did not have a previously defined host specialization.

Pangenome analysis and identification of cattle-specific core genes

Using Roary, we defined the pangenome amongst the 72 cattle strains and compared these data to the pangenome analysis of the 214 human strains (Chapter 3); a total number of 286 strains were compared. Among the 72 cattle strains, 4,997 unique genes were identified of which 536 were defined as core genes or were present in 99.0% of the cattle strains. These 536 core genes represent 10.7% of the *C. jejuni* pangenome (**Table 4.2**). A subset of 505 genes was defined as soft-core genes, while accessory genes made up 89.3% of the total genes identified in the 72 cattle strains. The accessory genome was further stratified into cloud genes (n=2791), shell genes (n=1165), and soft-core genes (n=505).

These gene stratifications differed from the more diverse pangenome defined by the totality of the human and cattle strains (n=286), which had 2.0 times more total genes (n=9,953) and 0.9 times fewer core genes (n=498) than the cattle strains alone. The core genome comprised 5.0% of the total pangenome, whereas the accessory genome represented 95.0%. Indeed, there were 0.5 times fewer genes in the accessory genome of cattle strains. Cattle associated isolates represented just a subset of the total *C. jejuni* pangenome.

Comparing the core-gene phylogeny and ANI PAM clustering methodologies

To assess the utility of the MiGA pipeline and ANI PAM clustering, we compared the maximum likelihood (ML) phylogeny of the 214 human isolates (Chapter 3) to the ANI PAM clustering tree generated using the neighbor joining algorithm through MiGA (**Figure 4.1**). This analysis demonstrated that the MiGA ML phylogeny was able to recapitulate the same clusters of strains representing Clades I-III defined in the core gene phylogeny (see Figure 5, Chapter 3), providing additional confirmation for the population structure defined among this set of human-derived *C. jejuni* strains. While descendant lineages such as those belonging to CC-61 within subclade IF, were separated based on ANI distances, these clustered separately from subclade IF in the ML phylogeny (indicated by orange circles in **Figure 4.1**).

ANI PAM clustering identifies related cattle and human *C. jejuni* strains

A midpoint rooted neighbor joining phylogeny based on ANI PAM clustering of all 286 cattle and human strains also resulted in a structure similar to the ML phylogeny of

615 core gene for the 214 human strains alone (see Figure 5, Chapter 3). Of note, the ANI PAM clustering separated the strains based on the seven gene MLST designation as well as previously defined host specialism associations (**Figure 4.2**). For example, strains belonging to the chicken specialist lineage, ST-353 within CC-21 of subclade ID in the core-gene phylogeny, grouped together amongst other strains defined as subclade ID.

The 72 cattle strains were identified within different areas of the phylogeny, though several clustered together within specific branches. Most of the strains separated into clusters according to the previously defined cattle specialist lineages as well, though a few exceptions were noted. Differences in strain clustering by farm were also observed as strains isolated from the dairy farm grouped together with chicken specialist lineages, such as ST-5538 within CC-354, which was not found in the two beef farms. Cattle specialist lineages such at ST-1244 within CC-61 representing strains from both cattle and humans, however, clustered together, whereas the ST-982 CC-21 strains from both sources could be differentiated from each other and clustered separately. Notably, several cattle strains belonging to ST-982 (n=8) and ST-459 (n=26) clustered among at least one other human strain belonging to the same ST.

High quality SNP (hqSNP) analysis differentiates cattle and human CC-61 strains

Fourteen CC-61 strains, including four human and 10 cattle derived strains, were selected for hqSNP analysis. Of note, all seven ST-1244 cattle strains clustered together with 100% bootstrap support despite being isolated from different farms in the same year; six were isolated from a beef farm in Ingham County and one was from a dairy farm in Clinton County (**Figure 4.3**). These seven strains had no SNP differences and all were resistant to ciprofloxacin and tetracycline; one strain was classified as multidrug resistant

in that it also had resistance to the macrolides. The two pan-susceptible cattle strains with the related STs, 61 and 3351, were further separated from the remaining 12 isolates.

Although the human strains belonging to ST-1244 clustered separately within the phylogeny with 97% bootstrap support, roughly 191 to 1,195 SNPs separated the human genomes from the cattle genomes. Within the two small human strain clusters, strains TW19149 and TW19278, which were both recovered from patients in 2014, only had 8 SNP differences and clustered together with 100% bootstrap support. Human strains TW19100 and TW16478 isolated in 2013 and 2011, respectively, were more distinct from each other with 96 SNP differences. All four of these human strains were resistant to tetracycline and were recovered from patients residing in three different counties.

Closely related ST-8 strains were detected in humans and cattle by hqSNP analysis

Five *C. jejuni* strains representing ST-8 of CC-21 were examined; four were recovered from human patients residing in four different counties and one was from a dairy cow in another county (**Figure 4.4**). The cattle strain US975 clustered together with human strain TW16510 with 100% bootstrap support and only differed by 88 SNPs; the cattle strain was not resistant to tetracycline as the human strain was, but both were recovered in 2012. Two highly similar human strains, TW16452 and TW16444, also clustered together with 100% bootstrap support but only had 2 SNP differences. These two strains were separated from the cluster containing the cattle strain (US975) and human strain (TW16510) and differed by ≥153 SNPs. TW19282, which was recovered

from a patient 2-3 years after the other ST-8 strains were isolated, was the most unrelated strain differing by ≥ 219 SNPs relative to the strains in the other clusters.

Recovery of closely related ST-459 cattle strains from different farms

Roughly 36.1% (n=26) of cattle strains were classified as ST-459 and ANI PAM clustered one human strain with these cattle strains; hence, we performed an hqSNP analysis for all 27 strains (**Figure 4.5**). Through this analysis we detected three larger clusters containing most (n=21) of the cattle strains that were separated from six other strains with 100% bootstrap support.

The first cluster contained seven identical strains with 0 SNP differences. These strains were recovered from different cattle residing in one Ingham County feedlot and were highly similar to 14 other strains recovered from cattle residing in a different feedlot located in Calhoun County. This group of strains represented two small clusters that differed by only 1 SNP. Overall, the cattle strains from the two counties, which were collected in the same year and were resistant to tetracycline, were separated by only 5-58 SNPs.

Another small cluster containing two cattle strains isolated from the same Ingham County feedlot had 100% bootstrap support and 0 SNP differences, though it was separated from the larger cluster by ≤1,085 SNPs. Although the human strain, TW16446, did not cluster with any of the cattle strains, it only differed from the closest cattle cluster by 729 to 2,029 SNPs.

Human and cattle derived ST-929 and ST-982 strains are distinct

Ten ST-929 strains recovered from an equal number of humans and cattle were also evaluated for hqSNPs. Although the strains from both sources were separated and formed two clusters in the phylogeny, one human derived stain only differed from cattle strains by 33 SNPs (**Figure 4.6**). When examined separately, the cluster of human strains differed from one another by 7 to 987 SNPs, whereas the cattle strains were separated into two clusters comprising highly related strains (0 SNPs) from two different beef farms. It is notable that the cattle strains in these two clusters could only be differentiated by 2 SNPs despite their recovery from different feedlots.

Similarly, 23 ST-982 strains, including eight from cattle and 15 from humans, were evaluated given their placement together within the ANI PAM phylogeny. The hqSNP analysis also identified unique human and cattle clusters that were separated from one another by >200 SNPs (**Figure 4.7**). Eight tetracycline resistant ST-982 strains isolated from different beef cattle in the same Calhoun County farm were highly related with 0 SNP differences. By contrast, three small clusters of human strains were identified with five strains representing singletons found on a separate branch in each cluster. Several groups of human strains within these clusters were highly similar. For example, TW16694 and TW16646, which were both resistant to tetracycline and identified in 2012, had 2 SNPs differentiating them; these two strains were separated from the cluster containing 11 human strains and the eight cattle strains by >100 SNPs. Within the latter cluster, five closely related human strains (11-36 SNPs) recovered from multiple years and counties with varying antibiotic susceptibility profiles were separated from the other strains by 210 to 770 SNPs. The cluster of human strains that were most closely related to the cattle

strain cluster was highly similar to each other but differed from the cattle strains by up to 2,144 SNPs. This cluster contained two ciprofloxacin and tetracycline resistant strains recovered in 2014 with only 2 SNP differences.

DISCUSSION

C. jejuni has a diverse host range and thus, the risk of human disease has been linked to a wide range of risk factors. A key transmission mode is via contaminated food products such as poultry and unpasteurized milk, or through indirect or direct contact with environmental reservoirs (3). As cattle have become increasingly linked to outbreak-associated campylobacteriosis in the US, we sought to understand the genomic diversity of strains from cattle and make comparisons to strains recovered from patients with *C. jejuni* infections during an overlapping time period (9, 45, 46). Not surprisingly, the strains identified from cattle were less diverse than the strains recovered from patients. Although fewer cattle strains (n=75) were examined, only 15 unique STs were identified relative to the 87 STs identified among the 214 human strains (Chapter 3). New alleles for the seven-gene MLST scheme were also identified in the cattle strains, leading to the identification of three new STs.

The pangenomic analyses identified a more restricted diversity among the cattle strains as the accessory genome was 89.3% compared to 95% for both the human and cattle strains combined. These data suggest that gene loss within some cattle specialist lineages may be occurring, which consistent with the identification of multiple cattle-associated lineages. Indeed, previous analyses have identified significant gene loss amongst emerging cattle specialist lineages (13). Loss of important genes could restrict

the ability of some lineages to be transmitted to and survive in different hosts and environments.

ANI PAM clustering of all 289 strains demonstrated that closely related strains from cattle and humans were circulating in Michigan during a similar time period. This finding indicates that a subset of strains with unique traits may be more readily transmitted to residents, particularly those with important risk factors such as livestock exposure, that can increase the likelihood of transmission. Given the proximity of these highly related human and cattle strains on the phylogeny, an hqSNP analysis was performed that could more accurately differentiate the *C. jejuni* strains from each other.

The hqSNP analysis of the seven strains belonging to CC-61, which represented a cluster of highly related cattle derived strains, were identical despite being recovered from two farms in the same year. One strain within this highly related cluster was isolated from a dairy farm, which was at least 30 miles away from the beef farm where the remaining six strains were isolated. This finding highlights the widespread distribution of a specific CC-16 lineage, suggesting enhanced adaptation to the cattle reservoir as well transmission. Because these strains differed from the cluster of CC-61 strains from human patients by up to 1000 SNPs, more comprehensive analyses are needed to determine whether the human strains have acquired important genes that may be essential for disease.

In addition, the hqSNP analysis identified several small clusters within the human CC-61 strains; two strains had only 8 SNP differences, which is interesting as they were from residents of the same county who acquired the disease in the same year. Hence, it is possible that these highly related clusters include strains from individuals with similar

exposures. In contrast, another cluster of five human derived CC-61 strains differing by 11-36 SNPs was isolated from patients in different years throughout the four-year period. Because these strains were also recovered from patients in different counties, it is possible that a sustained reservoir or routine exposure is important for infection.

Highly similar strains were also observed within ST-459, as three large clusters of 7-9 strains were identified. There were no intra-cluster differences between different strains, while 1 SNP separated clusters within farms and 5-58 SNPs separated clusters from differing farms. Additionally, amongst strains from ST-929 two small clusters of highly related cattle isolates were identified separating isolates from different farms by only 2 SNPs. These highly related cattle strain clusters further suggest that *C. jejuni* populations can be shared amongst differing host-biogeography within the same region.

Importantly, within ST-8 a dairy cattle strain was clustered together with a human strain that was identified within the same year and only had 88 SNP differences, further suggesting that highly related strains are circulating within cattle and that a subset of strains can cross over and cause human disease. Identifying which bacterial features are most important for transmission and disease, however, is critical for future surveillance efforts. Indeed, these data further emphasize the importance of monitoring strains from a variety of sources including reservoirs, food products, and patients with infections. Additionally, a more comprehensive evaluation of specific *C. jejuni* sub-populations and lineages may lead to new insights that could support more detailed epidemiologic studies and interventions.

APPENDIX

Supplementary Table 2 Genomic data for the 75 cattle-derived *Campylobacter jejuni* strains examined**.**

Table 4.1 Distribution and frequency of multilocus sequence types (STs), clonal complexes (CCs), and host associations among 72 cattle derived *Campylobacter jejuni* strains.

Sequence Types (STs)	**No.**	**(%)**	**Strain ID**
Singleton ST	12	(16.7)	ST-8 (US0975), ST-21 (US1131), ST-58 (US0972), ST-61 (US0782), ST-267(US0857), ST-922 (US0992), ST-3351 (US1071), ST-7689 (US0913), ST-7693 (US1015), new_6* (US1005), new_7* (US1109), new_8* (US1124)
ST-42	2	(2.8)	US0809, US0893
ST-459	26	(36.1)	US0940, US0984, US1016, US1028, US1034, US1036, US1040, US1044, US1055, US1058, US1060, US1061, US1070, US1076, US1078, US1081, US1087, US1096, US1112, US1113, US1114, US1119, U1120, US1129, US1133, US1134
ST-806	2	(2.8)	US903, US1006
ST-929	5	(6.9)	US1039, US1050, US1059, US1086, US1125
ST-933	3	(4.2)	US0900, US0992, US1003
ST-982	8	(11.1)	US1065, US1067, US1069, US1088, US1105, US1118, US1121, US1132
ST-1244	8	(11.1)	US0957, US0977, US0987, US0988, US0991, US0994, US0996, US1048
ST-5538	4	(5.6)	US0897, US0928, US0968, US0970
ST-7679	2	(2.8)	US0886, US0949
Total	72	(100)	

Clonal Complex (CCs)	**No.**	**(%)**	**STs**
Unassigned	6	(8.3)	ST-58 (n=1), ST-922 (n=1), ST-7689 (n=1), new_6 (n=1), new_7 (n=1), new_8 (n=1)
CC-21	15	(20.8)	ST-8 (n=1), ST-21 (n=1), ST-806 (n=2), ST-982 (n=8), ST-7679 (n=2), ST-7693 (n=1)

Table 4.1 (cont'd)

CC-42	28	(38.9)	ST-42 (n=2), ST-459 (n=26)
CC-61	10	(13.9)	ST-61 (n=1), ST-1244 (n=8), ST-3351(n=1)
CC-257	5	(6.9)	ST-929 (n=5)
CC-283	1	(1.4)	ST-267 (n=1)
CC-354	4	(5.6)	ST-5538 (n=4)
CC-403	3	(4.2)	ST-933 (n=3)

Host Specializations	**No.**	**(%)**	**CCs/STs**
Undefined	6	(8.3)	ST-58 (n=1), ST-922 (n=1), ST-7689 (n=1), new_6 (n=1), new_7 (n=1), new_8 (n=1)
Cattle	52	(72.2)	CC-21 ST-8 (n=1), CC-21 ST-806 (n=2) , CC-21 ST-982 (n=7), CC-42 (n=28), CC-61(n=10), CC-403 (n=3)
Chicken	10	(13.9)	CC-257 (n=5), CC-283 (n=1), CC-354 (n=4)
Generalist	4	(5.6)	CC-21 (n=4)

Table 4.2 Pangenomic gene categorizations for 72 cattle and 214 human derived *Campylobacter jejuni* strains as defined by Roary pangenome pipeline

Total Human and Cattle Pangenome (n=286)	**No.**	**(%)**	**Proportion as compared to cattle dataset (n=72)**
Core-genes [a]	498	(5.0)	0.93
Accessory Genome (AG) [b]	9455	(95.0)	1.89
Total Genes	9953	(100)	1.99
AG: Soft-Core Genes [c]	414	(4.2)	0.82
AG: Shell Genes [d]	1234	(12.4)	1.06
AG: Cloud Genes [e]	7807	(78.5)	2.80
Cattle Pangenome (n=72)	**No.**	**(%)**	**Proportion as compared to total dataset (n=286)**
Core-genes [a]	536	(10.7)	1.08
Accessory Genome (AG) [b]	4461	(89.3)	0.48
Total Genes	4997	(100)	0.50
AG: Soft-Core Genes [c]	505	(10.1)	1.22
AG: Shell Genes [d]	1165	(26.1)	0.94
AG: Cloud Genes [e]	2791	(55.9)	0.36

[a] Core-genes are defined as genes identified in ≥ 99% of the dataset
[b] the accessory genome (AG) is defined as all the genes outside of the core-genome. The accessory genome can be broken down into three components made of genes defined as soft-core genes, shell genes and cloud genes

Table 4.2 (cont'd)

[c] Soft-core genes are genes found in ≥ 95% of strains but less than 99% of strains

[d] Shell genes are defined as genes found in ≥ 15% of strains but < 95% of strains

[e] Cloud genes are observed in < 15% of strains

Figure 4.1 Comparative analysis of 214 *Campylobacter jejuni* strains isolated from gastroenteritis cases in Michigan based on a: **A)** 615 core gene alignment phylogenetic reconstruction in RaXML with 100 bootstrap replicates and rooted at midpoint; and a **B**) ANI PAM neighbor joining phylogeny. Connections are drawn to the same strains that were examined using both methods. Tip colors represent the clades and subclades as defined by >80% bootstrap in the core gene phylogeny (panel A). Blue represents clade I, red; Clade II, gray; subclade IA, light blue; subclade IB, orange; subclade IC, pink subclade ID, turquoise subclade IE, green subclade IF.

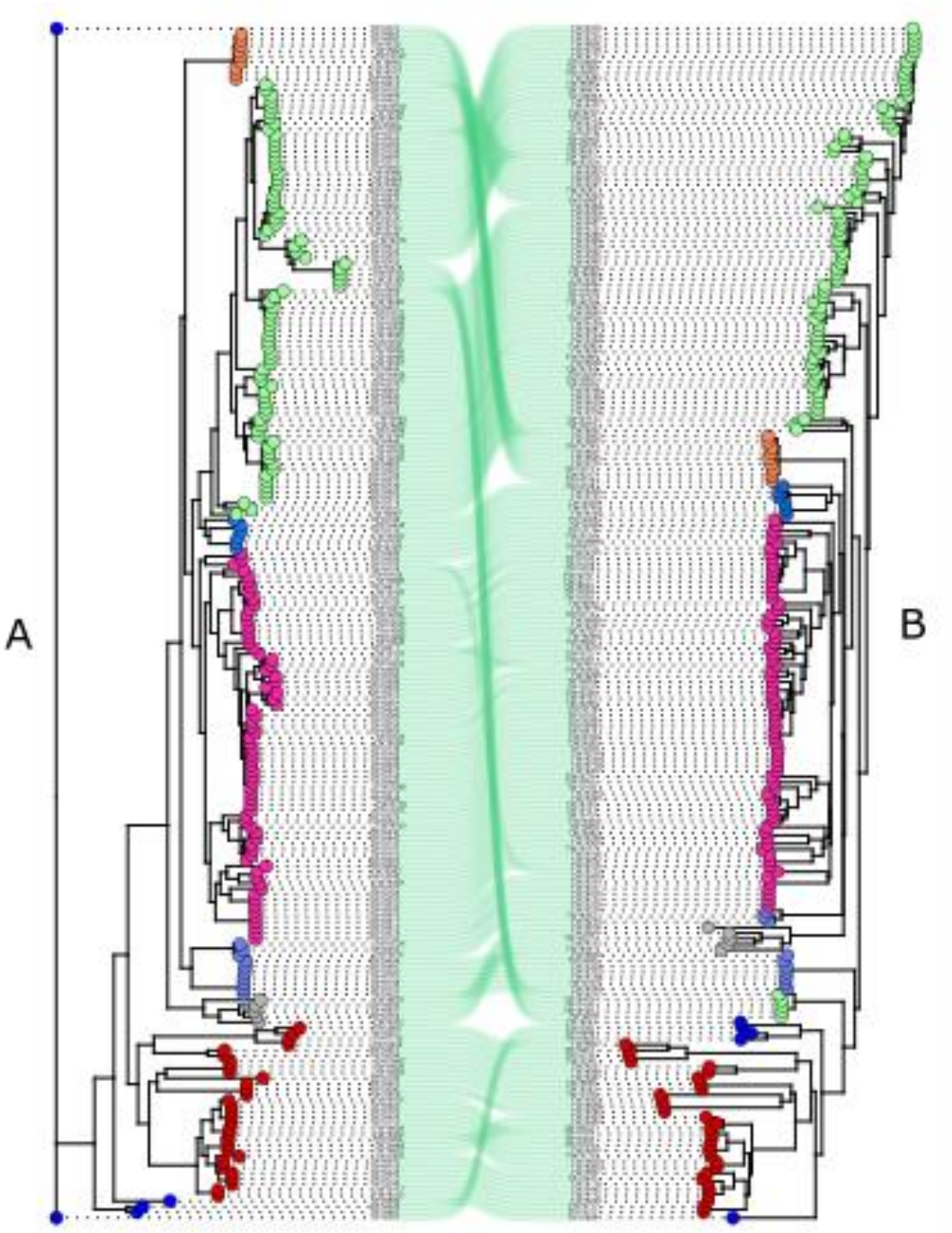


Figure 4.2 ANI PAM distance neighbor joining phylogeny annotated with strain source, multilocus sequence type (ST), clonal complex (CC), and previously defined host specialism associations

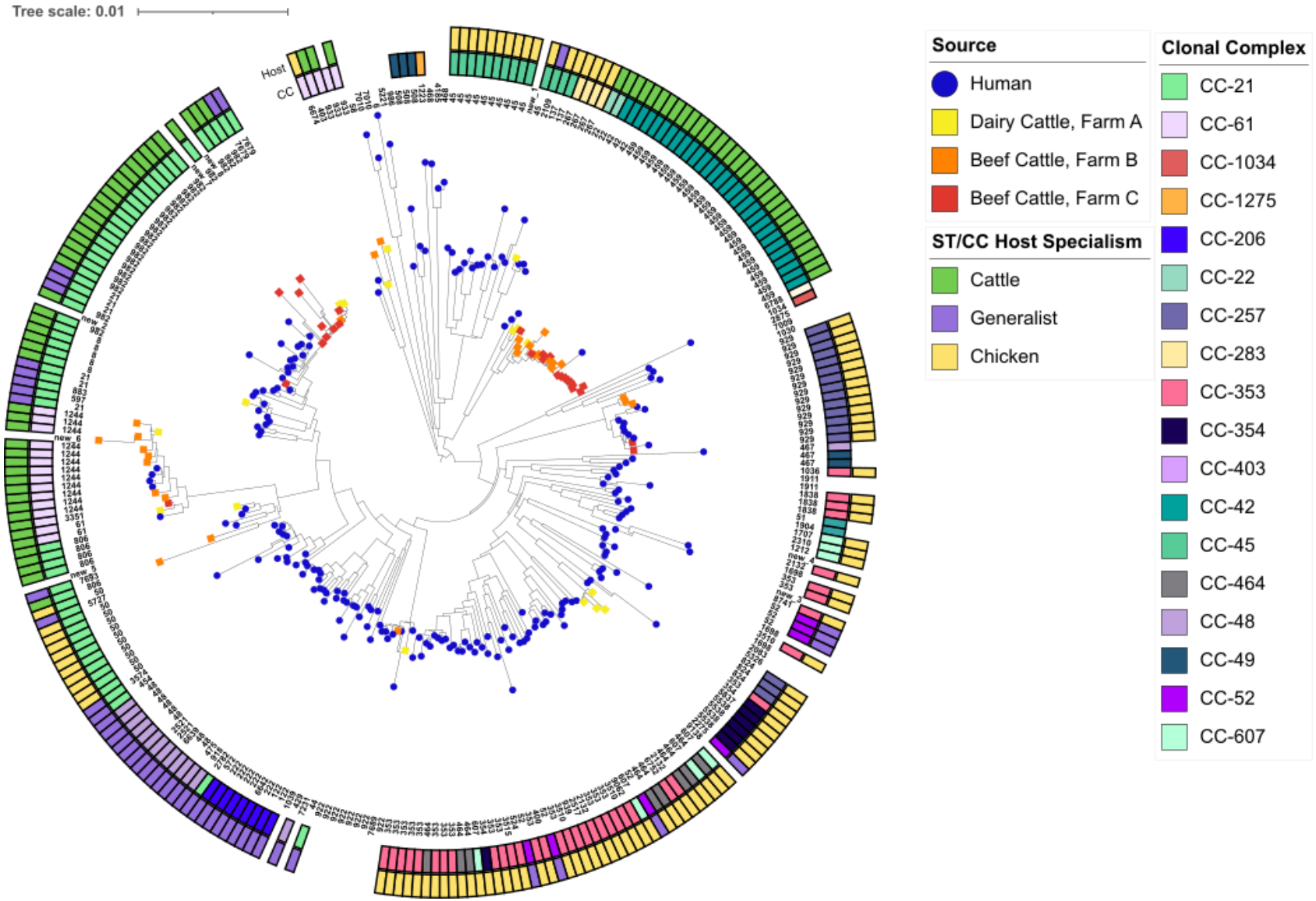


Figure 4.3 hqSNP maximum likelihood phylogeny of CC-61 rooted a midpoint annotated with source, antibiotic resistance phenotype, year of isolation and county of residence

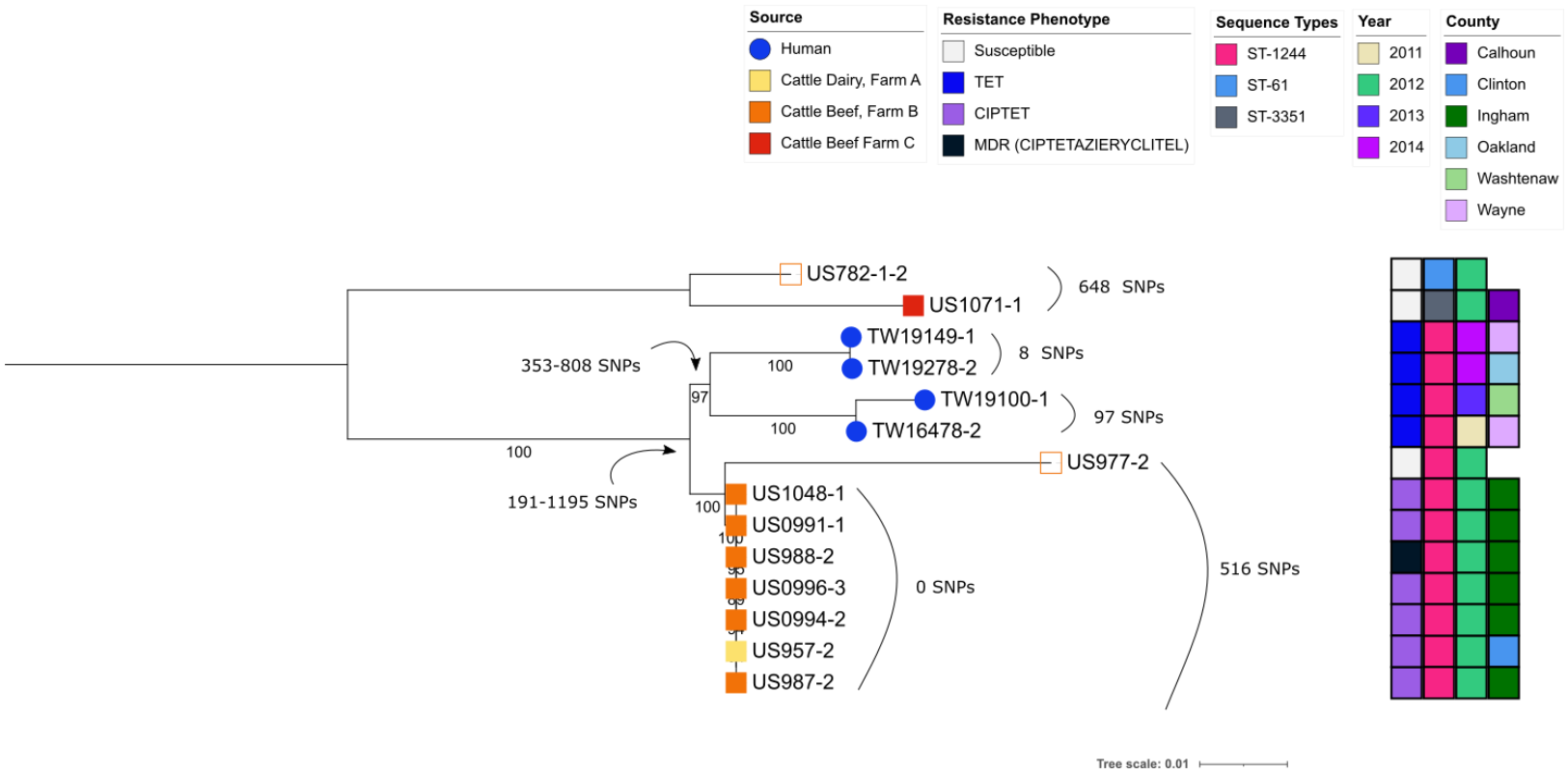


Figure 4.4 hqSNP maximum likelihood phylogeny of ST-8 rooted a midpoint annotated with source, antibiotic resistance phenotype, year of isolation and county of residence

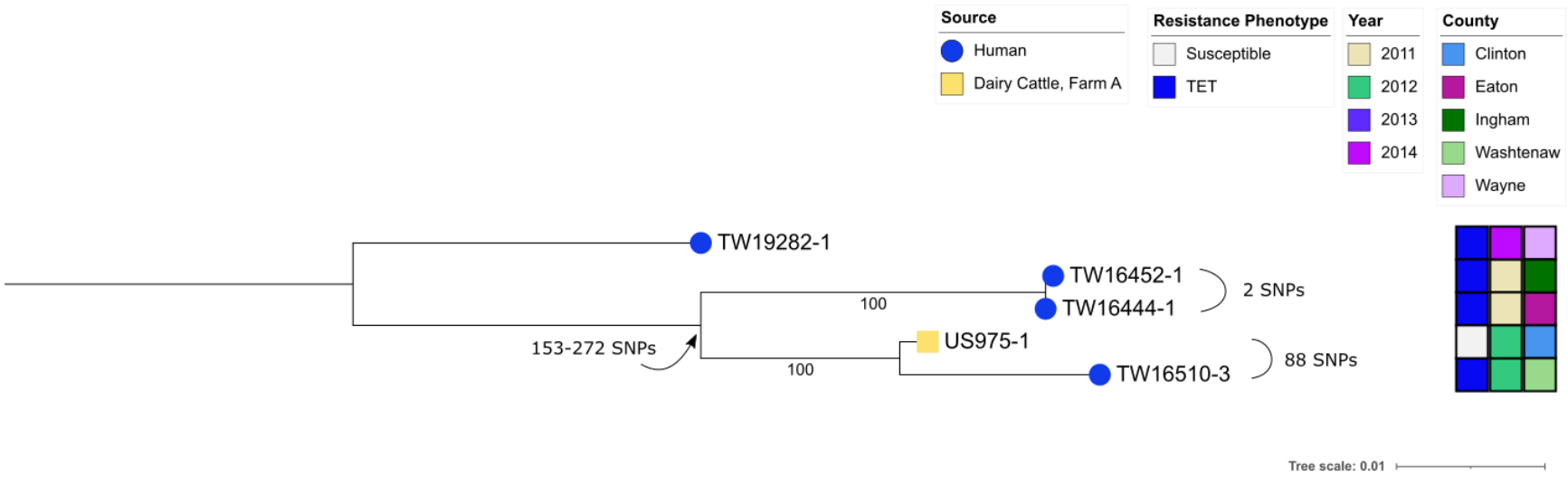


Figure 4.5 hqSNP maximum likelihood phylogeny of 27 ST-459 strains rooted a midpoint annotated with source, antibiotic resistance phenotype, year of isolation and county of residence

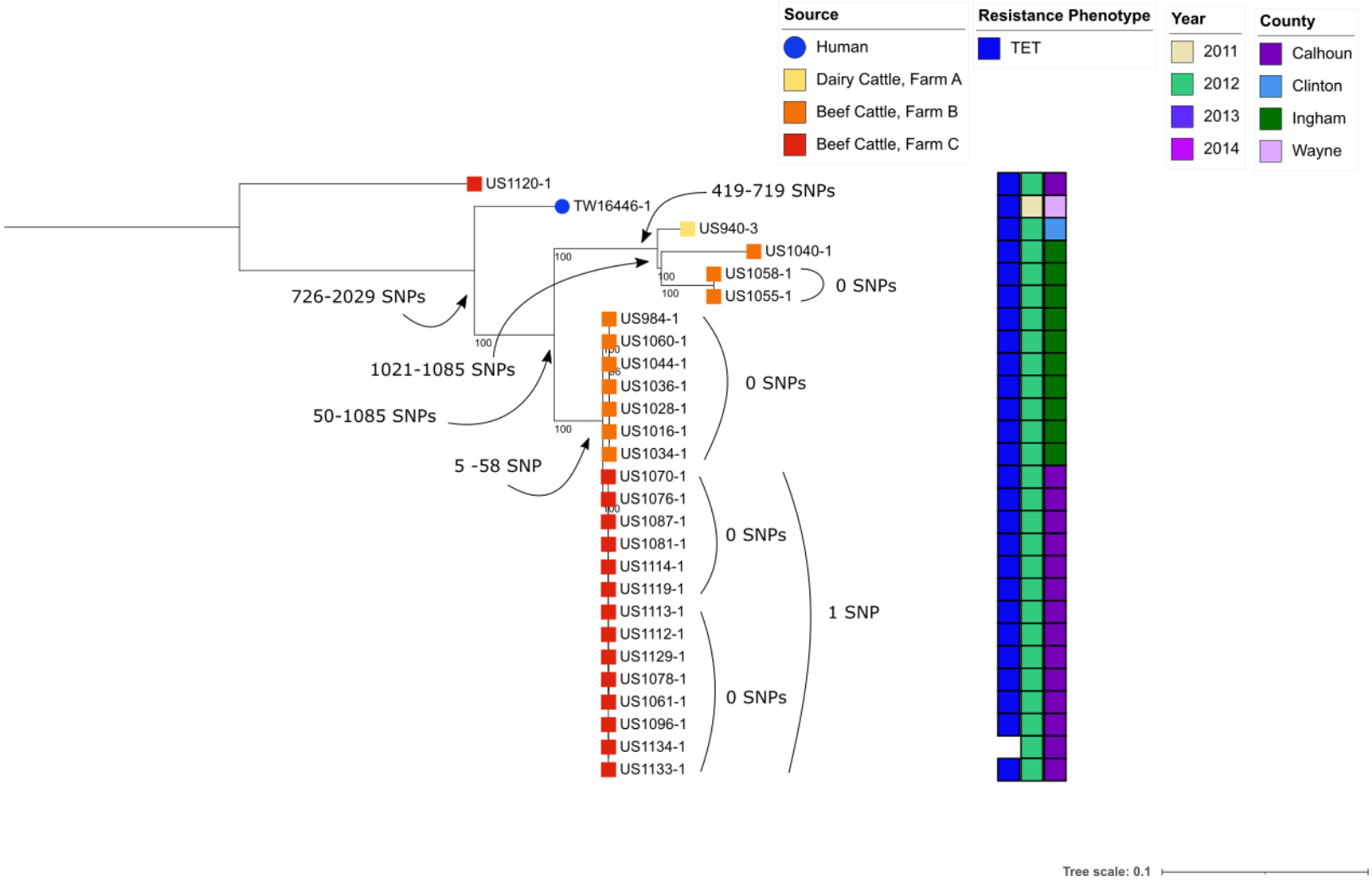


Figure 4.6 hqSNP maximum likelihood phylogeny of ST-929 rooted a midpoint annotated with source, antibiotic resistance phenotype, year of isolation and county of residence

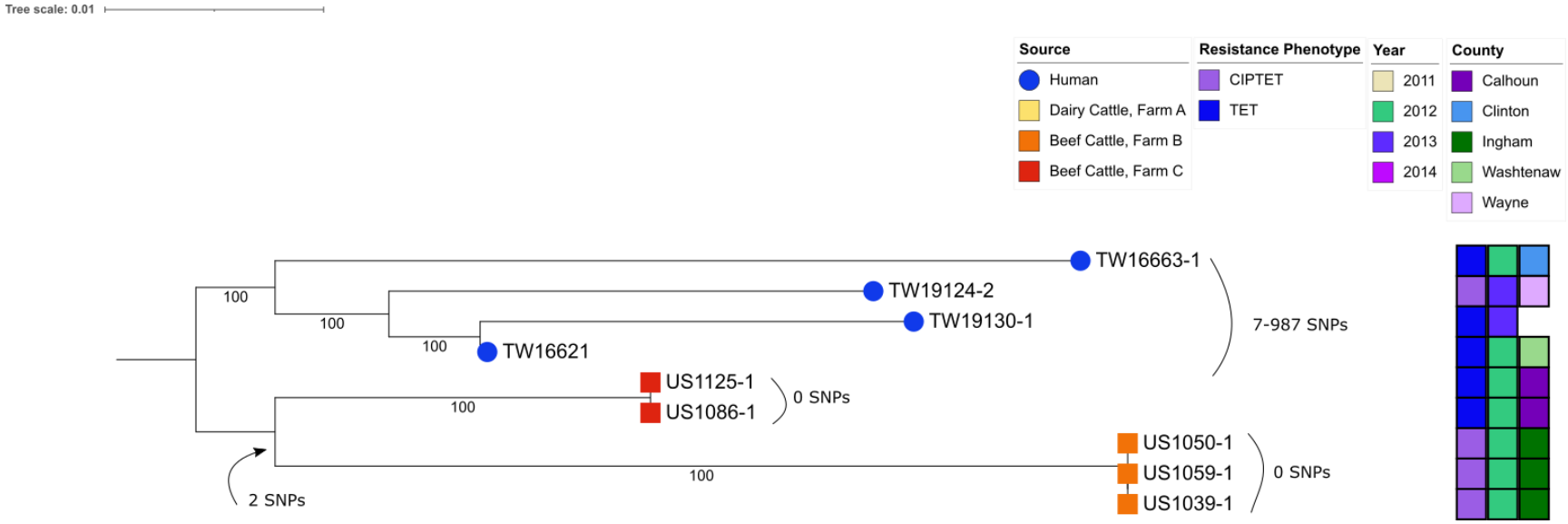
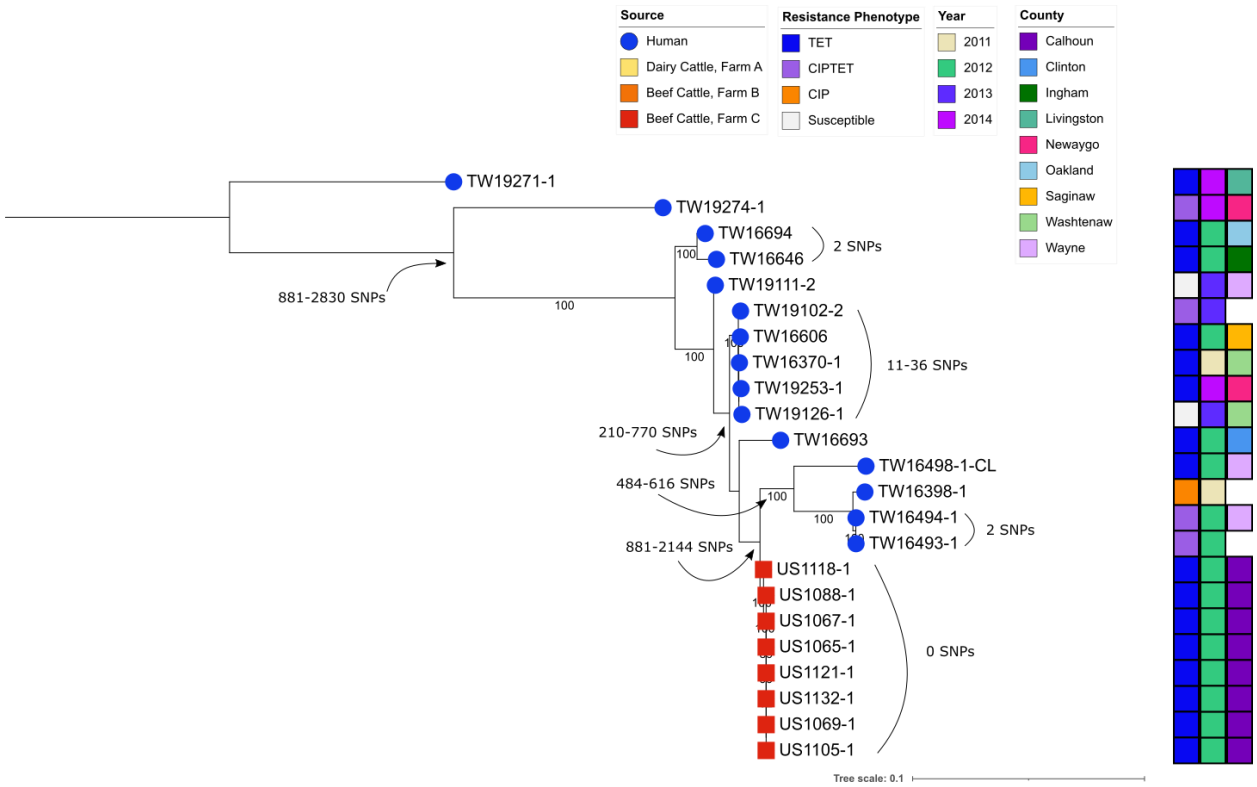


Figure 4.7 hqSNP maximum likelihood phylogeny of ST-982 rooted a midpoint annotated with source, antibiotic resistance phenotype, year of isolation and county of residence



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CHAPTER 5: FUTURE DIRECTIONS AND CONCLUSIONS

Campylobacter jejuni is the leading cause of foodborne illness in the US (1). The pathogen has a diverse set of ecological hosts including chicken, cattle, pigs, and wild birds (2). Additionally contaminated environmental reservoirs are also sources for indirect and direct transmission to humans. The National Antimicrobial Monitoring System (NARMS), which monitors antibiotic resistance of pathogens including *C. jejuni*, has reported antibiotic resistant *C. jejuni* as a serious public health threat (3). While resistance frequencies of *C. jejuni* are monitored for national trends, NARMS is not representative of the entire US population and does not enable accurate comparisons to be made between states that are not included in the system. Michigan is not a part of NARMS or FoodNET, the national surveillance system that monitors foodborne illness in the US (4).

Therefore, through an active surveillance system in Michigan, we examined a population of *C. jejuni* isolates recovered from patients with campylobacteriosis diagnosed between 2011 and 2014 to assess frequencies of and identify associations with antibiotic resistant infections (Chapter 2). Ciprofloxacin and tetracycline resistant infections were the most clinically important and predominant resistance phenotypes identified, respectively. Individuals with ciprofloxacin resistant infections were more common in patients who had traveled in the past month, whereas tetracycline resistant infections were more common among patients reporting contact with livestock or consumption of well water. We also found that age >40 years, international travel in the past month, and non-white race were associated with hospitalization. These data suggest disparities between patient populations and highlight the need to more comprehensively examine bacterial characteristics across locations. The use of socially constructed variables such as race, ethnicity, and socioeconomic status is useful in describing

preliminary evidence of differences between populations however these proxy measures do not address causal epidemiologic questions related to health disparities. Examining associations between disease severity and neighborhood level factors, such as resource deprivation, and food dessert status, could further examine the impact of racism rather than race as a risk factor for hospitalization due to *C. jejuni* infection (5).

Overall, our data demonstrated that resistance frequencies in *C. jejuni* differed in Michigan relative to those reported nationally via NARMS, with higher levels of tetracycline resistance frequencies in Michigan. In Chapter 2 we further demonstrated that risk factors for ciprofloxacin resistant infections included a recent history of international travel and that these infections were more likely to result in hospitalization than tetracycline resistant infections. These data highlight the importance of monitoring bacterial populations and associated risk factors for disease, which can be later used to inform public health action.

In Chapter 3 we analyzed the 214 strains from Michigan patients by whole-genome sequencing (WGS) to determine the diversity and population structure of circulating *C. jejuni* strains. A pangenomic analysis of 615 core genes demonstrated remarkable diversity among the strains that represented 87 unique multilocus sequence types (STs) belonging to three clades; Clade I comprised six subclades, IA-IF. Several groups of highly related strains were also observed, suggesting similar exposures within the population over the time period. Notably, cases reporting recent travel had greater genomic diversity than those who reporting no recent travel. Specific lineages, such as subclade ID, which was associated with ciprofloxacin resistance, also increased over the 4-year period, and key antibiotic resistance determinants were detected among the

resistant strains. Differences in the distribution of some lineages were also observed between urban and rural residents, suggesting variable risk factors by residence. We also identified the key mechanisms linked to antibiotic resistance *C. jejuni* in Michigan, and uncovered evidence of clonal expansion, and co-selection of resistance determinants within a variety of environments. Action toward reduction of antibiotic resistance could be supported by further pangenomic epidemiologic studies that examine lineages carrying clinically important resistant determinants. Furthermore, use these data to conduct bacterial genome wide associations studies along with examining virulence gene composition and associations with severe disease could lead to new insights related to pathogenesis. More specifically, the diversity of virulence genes and allele frequencies within this pathogen population could impact disease presentation.

Since 15.9% of the *C. jejuni* strains from patients with gastroenteritis were classified as cattle-specialist lineages in Chapter 2, we sought to compare these strains to 72 *C. jejuni* strains from cattle in three different herds in Chapter 3. WGS analyses identified clusters of highly related strains, with the cattle strains clustering together with each other or specific lineages of human strains. Interestingly, there were highly related strains from different beef farms in some cases where only 2 SNPs were identified. Furthermore, small clusters of highly related human strains were also identified. These phylogenetic comparisons highlight the presence of co-circulating strains in different cattle operations while some highly similar strains were linked to campylobacteriosis in humans.

Herein, we have illustrated that *C. jejuni* is a diverse pathogen as strains isolated from patients have a variety of different host associations. Importantly, the use of WGS

and pangenome approaches has demonstrated that patients are being exposed to highly similar as well as divergent pathogen populations that are ultimately shaped by recombination. While comparative genomic analyses have demonstrated highly similar strains circulating in cattle and humans. Although, these studies were unable to determine whether there was direct or indirect transmission between cattle and humans, they do illustrate the potential for interspecies transmission as highly similar strains could be found in both sources. Enhancing our understanding of the population structure and genomic diversity of circulating *C. jejuni* strains could give insights into the biological properties important for transmission and disease, and may lead to more accurate and specific public health actions led by real-time genomic epidemiologic inferences.

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