EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF CAMPYLOBACTER IN MICHIGAN

By

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ABSTRACT

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Campylobacter is a zoonotic agent and the leading cause of human gastroenteritis worldwide. An increasing trend for both campylobacteriosis incidence and antimicrobial resistance of *Campylobacter*, especially of *C. jejuni*, is reported globally. In the U.S., Center for Disease Control and Prevention (CDC) conducts surveillance systems in 10 sites to monitor the incidence and track the trends of antimicrobial resistance of *Campylobacter*, however, Michigan is not included in the system. This dissertation is dedicated to describe the epidemiology of campylobacteriosis in Michigan, and further characterize the antimicrobial resistance and genetic diversity using a subset of recovered human isolates. Furthermore, *C. jejuni* isolates from cattle were characterized for the genotypes and antimicrobial resistance, and compared with human isolates to elucidate the association, and possibly the transmission dynamics of *C. jejuni* between two species.

A descriptive epidemiology study was conducted using the data in Michigan Disease Surveillance System in 2004-2013; a total of 7,128 cases of campylobacteriosis were included. Although the average annual incidence rate was significantly lower than what has been reported for the nation, an increasing trend, especially in age groups of 20-29 years, and 50 years and above, was observed. Distinct seasonality in summer months, especially in July, was observed consistently over all years, and the trend was more prominent in mid age groups, i.e. 10-19 years, 40-59 years, implicating that specific behaviours may contribute to the seasonality. Age-adjusted incidence rates at the county level showed higher incidence of campylobacteriosis reported in rural areas compared to urban areas. The risk among cases from rural areas was significantly associated with livestock contact and drinking well water at home. Notably, an increasing trend of hospitalization rate due to campylobacteriosis was observed over time, and the risk was higher in >60 years of age, and also cases living in urban areas relative to rural areas.

To determine the frequency of antimicrobial resistance and the genetic diversity of *C. jejuni* in Michigan, 94 *C. jejuni* isolates were collected from patients at four Michigan hospitals in 2011-2012. A similar prevalence of fluoroquinolones and macrolides resistance was observed in the *C. jejuni* isolates as what has been reported for the nation. Fluoroquinolone resistance was significantly associated with foreign travel, as previously reported in the U.S., and other countries. A significantly higher prevlance of tetracycline resistant *C. jejuni* was found in Michigan, and the resistance was linked to multilocus sequence type (ST)-982, which was only recovered from livestock and farm environment in the U.S. previously. Furthermore, tetracycline resistant *C. jejuni* was significantly associated with livestock contact, suggesting livestock, i.e. cattle, as a potential reservoir for tetracycline resistant *C. jejuni* infections.

To better understand the ecology of antimicrobial resistant *C. jejuni* transmission in Michigan, 135 *C. jejuni* isolates recovered from three cattle farms in Michigan in 2012, were characterized for the antimicrobial resistance and genetic diversity. Significant associations between certain STs and the resistance profiles were observed; ST-459, the most prevalent ST among cattle *C. jejuni* isolates, was significantly associated with tetracycline resistance, while ST-1244 had significantly higher likelihood to be resistant to both fluoroquinolone and tetracycline. ST-982, which was linked with tetracycline resistance in human isolates was prevalent in cattle and most of the ST-982 isolates from cattle were resistant to tetracycline, suggesting cattle as an important source of tetracycline resistant *C. jejuni* infections in humans in Michigan. Seven additional STs were shared between humans and cattle, and all of the STs were more closely related to cattle-derived isolates in the phylogenetic analysis, warranting continous monitoring of the prevalence and antimicrobial resistance of *C. jejuni* in this important reservoir. To my beloved husband, Seungeun, and our precious daughter, Peet

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KEY TO ABBREVIATIONS

CC Clonal Complex

- CipNal Ciprofloxacin-, Nalidixic acid- resistant
- CipNalTet Ciprofloxacin, Nalidixic acid, Tetracylcine-resistant
- CLSI Clinical and Laboratory Standards Institute
- ECOFF epidemiological cut-off value
- **FoodNet** Foodborne Disease Active Surveillance Network
- **GIS** Geographic information System
- MDHHS Michigan Department of Health and Human Services
- MDR Multi-drug resistance
- MDSS Michigan Disease Surveillance System
- MIC Minimal Inhibitory Concentration
- NARMS National Antimicrobial Resistance Monitoring System
- PHI Pairwise Homoplasy Index
- **Rep-PCR** Repetitive Sequence-based Polymerase Chain Reaction
- **ST** Sequence Type

CHAPTER 1

Literature review: Epidemiology and antimicrobial resistance of *Campylobacter*

in the United States

INTRODUCTION

Campylobacter spp. are spiral shaped gram negative bacilli, which form the characteristic 'gull-wing' shape under the microscopy.¹ The organism was originally identified as a cause for ovine abortions in 1913, which was then described as a 'related *Vibrio*'.² After the first isolation of the bacteria from humans with diarrhea in 1957, selective culture media was developed in the 1970s, which greatly facilitated the recognition of *Campylobacter* as one of the most frequently isolated enteric bacteria by the 1980s.^{3,4} Currently, *Campylobacter* is the leading cause of human gastroenteritis worldwide, as defined by the World Health Organization.⁵ A high incidence of campylobacteriosis has been reported in developed countries with numbers as high as 1,512 cases per 100,000 population.⁶ A dramatic increase in the incidence has been observed in the last decade around the world, including North America, Europe, and Australia,⁷ and it is suggested to be even more prevalent in developing countries.^{5,7} The high incidence directly contributes to large costs, including medical expenses, lost wages, product recalls, legal costs, and other indirect expenses. In the U.S., the Centers for Disease Control and Prevention (CDC) estimates about 1.3 million human campylobacteriosis infections occur annually, costing about \$1.7 billion each year.8

Campylobacter infection causes diarrhea, abdominal pain, and fever within two to five days after exposure, and is often accompanied by other symptoms, e.g. bloody diarrhea, nausea, and vomiting.⁹ Most of the infections resolve by 7 to 10 days without medication. However, in some cases, especially in infants or individuals with compromised immune systems, *Campylobacter* can develop more severe infections including bacteremia and

septicemia, resulting in deaths.¹⁰ Furthermore, recent studies are showing significant associations between *Campylobacter jejuni* infections and auto-immune diseases like Guillain-Barré Syndrome (GBS),¹¹ reactive arthritis,¹² and chronic inflammatory conditions like inflammatory bowel disease,¹³ which contribute to a higher morbidity and economic impact.

C. jejuni is the most common species found in human *Campylobacter* cases. Studies have shown that *C. jejuni* colonizes the gastrointestinal tract in various animal species including chickens, cattle, pigs, and wild birds, without causing clinical signs.^{14–16} Nonetheless, *C. jejuni* is most commonly isolated from chickens with observed flock colonization rates up to 90%.^{17,18} The thermophilic property of the species, growth at 37-42°C, is thought to be the major factor contributing to the adaptation in chickens, which have a body temperature of 41-42°C.¹⁹ With the high prevalence and high consumption rate of chicken meat all over the world, eating and handling chickens and chicken meat have been identified as the major risk factor for human infections.^{20,21} Another risk factor for campylobacteriosis, which has been commonly described in different countries is foreign travel.^{22,23} In fact, campylobacteriosis was found to be the main cause of travel-associated diarrheal disease in North America and Europe in the last decade.^{24–26}

Foreign travel has also been significantly associated with the increasing trend of antimicrobial resistant *Campylobacter* infections, especially against fluroquinolones such as ciprofloxacin. Fluoroquinolones and macrolides, azithromycin and erythromycin, are antimicrobials that are the first line agents for treating campylobacteriosis. However, since the late 1980s, increasing trends of fluoroquinolone-resistant *C. jejuni* have been reported

in Europe^{27,28} and the U.S.^{29–31} In fact, a high prevalence of fluoroquinolone-resistant *C. jejuni* has been reported in various geographical locations, including South Africa,³² Thailand,³³ and Spain,³⁴ posing a high risk of fluoroquinolone-resistant *C. jejuni* infections for travelers. Macrolide-resistant *C. jejuni* has been observed less frequently,³⁵ although the use of macrolides at therapeutic or subtherapeutic concentrations in food-producing animals was suggested to be a risk factor for the emergence of macrolide-resistant strains.^{36–38} Because of the growing concern over the increasing incidence of *C. jejuni* infections as well as antimicrobial resistance frequencies, continuous monitoring of incidence trends and antimicrobial resistance profiles is warranted.

In the United States, the CDC maintains a population-based surveillance system, the Foodborne Disease Active Surveillance Network (FoodNet), to identify laboratoryconfirmed infections of nine foodborne pathogens: *Campylobacter, Cryptosporidium*, *Cyclospora, Listeria, Salmonella*, Shiga-toxin producing *Escherichia coli, Shigella, Vibrio*, and *Yersinia*.³⁹ FoodNet was initiated in 1996 as part of the Emerging Infections Program and is currently focusing on 10 states, which represents roughly 15% of the U.S. population (47.5 million persons). Additionally, the National Antimicrobial Resistance Monitoring System (NARMS) tests a subset of samples recovered via FoodNet for susceptibility to antimicrobials of human and veterinary medical importance in order to identify nationwide trends.⁴⁰ NARMS is operated via collaboration between the CDC, FDA, the U.S. Department of Agriculture (USDA), and the state and local health departments. The CDC tests bacterial isolates from humans, while the FDA and USDA tests isolates from retail meats and food animals, respectively. Since 1997, NARMS has been characterizing the

antimicrobial resistance profiles of *Campylobacter* isolates recovered from humans via FoodNet, while characterization of isolates recovered from chicken and retail meat began in 1998 and 2002, respectively.

This chapter represents a summary and review of incidence rates, risk factors, and clinical outcomes associated with *Campylobacter* infections in the U.S. using raw data from FoodNet reports (1997-2012) and from previous studies conducted at FoodNet sites. Additionally, we reviewed antimicrobial resistance trends for *Campylobacter* infections in the U.S. by examining data from prior studies and reports from NARMS while focusing on human-derived isolates and resistance to antimicrobials of clinical importance.

INCIDENCE AND EPIDEMIOLOGY OF CAMPYLOBACTER IN THE U.S., 1997-2012

Study population

Since the FoodNet surveillance system was initiated in the U.S., the population under surveillance increased from 20.3 million (7.5% of the U.S. population) in 1997 to approximately 47.8 million (15.2% of the U.S population) in 2012. When the surveillance started in 1997, there were five sited included: California, Connecticut, Georgia, Minnesota and Oregon. In 1998, it expanded to seven sites with the addition of Maryland and New York. In 2000 and 2001, Tennessee and Colorado were added to the surveillance, and by adding New Mexico in 2004, the surveillance comprised 10 distinct sites. One thing to note about the surveillance sites is that California, Colorado, and New York contribute only a subset of counties, while the remaining seven sites have state-wide surveillance efforts. Thus, it is possible that the incidence rates reported for California, Colorado, and New York are not representative of the entire population in each of these states. These factors should be considered when analyzing the total incidence as well as the difference between sites. To account for such site-to-site variation and changes in the size of the population under surveillance over time, FoodNet estimates the change of incidence of infections between years using a main-effects, log-linear Poisson regression model (negative binomial model).⁴¹

Incidence trend and epidemiology

In the first two years of surveillance, *Campylobacter* was the most frequently reported pathogen among the nine foodborne pathogens, even surpassing *Salmonella*. Between the years of 1997-1999, however, the incidence of *Campylobacter* dropped

dramatically (Figure 1.1.), which has been attributed in part to successful implementation of the Hazard Analysis Critical Control Points (HACCP) by the USDA.⁴² In detail, the HACCP program requires that meat and poultry companies increase efforts to sanitize plants, conduct microbiological testing, implement quality controls, and create standards to control contamination by pathogens.⁴³ Although the measures were aimed primarily at Salmonella and E. coli 0157, it also contributed to a decrease in Campylobacter contamination. Other factors including food safety education, on-farm pathogen reduction efforts, and improved restaurant practices were also thought to contribute to this decline. Notably, there were differences in the rate of decline by geographic location. In California, for instance, the incidence declined from 57.6 cases per 100,000 in 1996 to 32.2 cases per 100,000 in 1999, almost dropping by 44%. As the cases from California comprised 27–35% of the total cases reported during the time period, the decline in California significantly contributed to the total decline.⁴⁴ The incidence in Connecticut, Georgia, and Maryland also showed declining incidence trends, while the other FoodNet sites did not have any significant changes. Overall, the decline in incidence was sustained until 2009, and then it started to increase in 2010. In 2012, it was estimated that there had been a 13% increase of *Campylobacter* incidence in the U.S. compared to 2006-2008.⁴⁵ This increased incidence was sustained through 2014, and *Campylobacter* and *Vibrio* are the only pathogens, among the nine foodborne pathogens, that are showing increasing trends in FoodNet currently.

Because the site specific incidence rates were not immediately available in FoodNet reports, comparisons across geographic locations can only be made from 2005 to present. Between 2005 and 2012, varying trends of incidence were observed across sites. The

incidence of *Campylobacter* in California, Maryland, Oregon, for example, increased over the years, while the incidence in Colorado and New Mexico declined (Figure 1.2.). Despite these changes, the geographic variation in *Campylobacter* incidence was sustained throughout the surveillance period. The factors associated with this variation, however, are not fully understood. Since FoodNet conducts active surveillance and audits clinical laboratories routinely, it is unlikely that differences in reporting practices were important for the geographic variation. Furthermore, a survey conducted at the FoodNet sites also showed that most of the clinical laboratories (>97%) tested for *Campylobacter* routinely, using a culture method.⁴⁶ To investigate if there were differences in risk factors and medical care seeking or medical practices between sites, a case-control study was conducted at seven FoodNet sites in 1998-1999.⁴⁷ Investigators compared the frequency of exposure to risk factors for *Campylobacter* infection including eating chicken at a restaurant, contact with farm animals or animal stool, drinking water from a lake, river, or stream or unpasteurized milk between sites, but did not find any significant differences. Also, although some variation was identified, the proportions of individuals seeking medical care or stool sample submission practices were not significantly different between sites. Additionally, other enteric diseases, which share similar symptoms with campylobacteriosis, showed different geographic patterns, supporting that it was not surveillance artifacts or bias driving the geographic differences. Consequently, it was concluded that the geographic differences in campylobacteriosis incidence are real, and may reflect differences in the risk of illness across sites. These findings warrant the investigation of factors important for human infections at each site such as monitoring the

prevalence of *Campylobacter* in reservoir animals over time, and tracking changes in meat processing protocols at plants, markets, and slaughter houses at each site.

Distinct seasonality was observed in all years, with *Campylobacter* cases reported more frequently in summer months (June to August), peaking in July. The proportion of cases observed in the summer months was around 38% in most of years, except for 2006 and 2007, in which 54% and 44% of the total *Campylobacter* cases were reported in the summer months, respectively. Higher levels of poultry contamination in the warmer months and eating patterns in the summer, including barbecuing, and eating outdoors, have been discussed as possible explanations for the seasonality.⁴⁴

When stratified by age, the incidence rate for children <1 year of age was the highest during all years, ranging from 56.0 per 100,000 in 1997 to 24.4 in 2012. The incidence for this age group was significantly higher compared to other age groups in all years. A case-control study was conducted at eight FoodNet sites in 2002-2004 to identify the risk factors specific for this age group. For infants 0-6 months, drinking well water and riding in a shopping cart next to poultry or meat were identified as risk factors, while visiting or living on a farm, having a pet with diarrhea at home, and eating fruits and vegetables at home were risk factors for campylobacteriosis in infants 7-11 months of age.⁴⁸ Similarly, an increase in incidence was observed in adults over 20 years, and especially in adults over 60 years (Figure 1.3.). Accounting for under-diagnosis rates in the analysis, a study conducted using data from FoodNet from 1996-2012 showed that *Campylobacter* incidence in adults older than 65 years of age has been increasing steadily since 2005.⁴⁹ They also found that older adults were more likely to seek medical care compared to the general population.

However, within in the older adults group (>65 years), the incidence rate for *Campylobacter* declined with age as opposed to other enteric infections showing a greater risk as age increased. With the anticipated increase in the population of people over 65 years of age in the U.S., investigations are warranted to identify risk factors specific for this age group.

Differences in incidence rates have also been identified by sex. Specifically, the incidence in males was significantly higher than in females in all years across sites, with the incidence rate ratio ranging between 1.21 and 1.31 (*p*<0.0001). Similar findings were observed in other countries,⁵⁰ and because other enteric pathogens did not have similar differences by sex, it was suggested that some sex-specific risk factors may be important for campylobacteriosis.⁵¹ Behavioral differences associated with food handling, preparation, and consumption may partly explain the higher risk observed in males, however, the difference persists even among young children and infants. For example, young boys including infants were observed to have a greater incidence of other infectious diseases (e.g., salmonellosis and shigellosis), suggesting that males may have greater susceptibility to infectious diseases. It is possible to speculate that there may be sex-specific differences in immunity, warranting a further investigation.

Other risk factors have also been described to be associated with sporadic campylobacteriosis in the U.S. Examples include foreign travel, direct and indirect animal contact with animals, consuming certain food items including raw milk, chicken, turkey, non-poultry meat at a restaurant, and raw seafood.^{22, 52, 53} However, other than a recent study on foreign travel,²² the data used in the studies were collected between 1998 and

1999, demonstrating a considerable time gap to apply the findings to current incidence trends. Given that more sites have been included in the FoodNet system and the nationwide increases in incidence trends, additional epidemiologic investigations are warranted to identify risk factors in each site and across all sites combined.

Clinical outcomes

FoodNet also records hospitalization and mortality rates associated with each enteric pathogen by year. Only hospitalizations occurring within seven days of the specimen collection date are recorded, and the survival status is determined at discharge or seven days after the collection for outpatients. Importantly, there has been a general increasing trend in hospitalization rates, from 10% in 1997 to 17% in 2014 (Figure 1.4.). When stratified by age, hospitalization rates were highest in the elderly, especially age 70 and above (Figure 1.5.). Interestingly, based on the FoodNet reports during 2008-2012, the hospitalization rate decreased over time for the group of 70-79 years, while the rate for 80 years and above showed an increasing trend. *Campylobacter* was more likely to be isolated from the blood in older adults compared to the general population (3% versus $\leq 1\%$), suggesting a greater likelihood of more severe infections as age progresses. ^{49, 54, 55}

A total of 120 deaths were reportedly attributable to *Campylobacter* infection during the 18 years of surveillance. The case-fatality rate (CFR), which is calculated by dividing the number of deaths by the total case numbers each year and multiplying by 100, has been estimated since 2008. The overall rate ranged between 0.06% and 0.2%, without any apparent trends over time. A study that used FoodNet data reported an increasing CFR with age from 0.2% at 65-69 years to 1.2% at 85 years and above.⁵⁶ However, it was

suggested that the high rates of hospitalization and mortality observed in the elderly was likely to be confounded by the high prevalence of comorbidities.^{57,58} The increased risk for severe outcomes along with the high cost of treatment indicate that individuals in this age group could benefit from more effective food safety interventions.⁵⁶

Studies have also been conducted at several FoodNet sites to investigate the longterm consequences of campylobacteriosis such as reactive arthritis and inflammatory bowel disease.^{59,60} A study in California between 1998 and 1999, for example, found that 8.6% and 2.8% of all *Campylobacter* cases developed persistent gastrointestinal symptoms and rheumatologic symptoms, respectively.⁵⁹ Meanwhile, a study in Minnesota and Oregon conducted between 2002 and 2004 reported 2.1 cases of newly developed reactive arthritis per 100,000 of *Campylobacter* cases.⁶⁰ Another study conducted in Oregon using hospital discharge data from 1997 to 2003 reported a declined incidence of GBS following the decline in *Campylobacter* incidence during the time period, confirming the association between two diseases. Further investigations to address the specific risk factors for developing these long-term sequelae among *Campylobacter* cases are needed.

ANTIMICROBIAL RESISTANCE OF CAMPYLOBACTER IN THE U.S., 1997 – 2012

Sample collection and susceptibility testing methods

Given the global concern about increasing resistance frequencies in *Campylobacter* isolates, NARMS expanded the collection sites from five states (California, Connecticut, Gerogia, Minnesota, Oregon) in 1997 to 10 FoodNet sites in 2003. From 1997 to 2004, each participating site forwarded the first isolate received in each week to the CDC for testing. Starting in 2005, each public health laboratory at all 10 sites forwarded representative isolates to the CDC based on the calculated burden of *Campylobacter* in each site using FoodNet data. For instance, all isolates received by Georgia, Maryland, New Mexico, Oregon, and Tennessee were forwarded, while every other isolate from California, Colorado, Connecticut, and New York, and every fifth isolate from Minnesota were tested. With the expansion of FoodNet surveillance, the number of human isolates tested increased over the years, from 209 and 4 isolates of *C. jejuni* and *C. coli* in 1997 to 1,191 and 134 isolates in 2012, respectively.

From 1997 to 2004, eight antimicrobials including azithromycin, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, gentamicin, nalidixic acid and tetracycline, were tested. In 2005, telithromycin was added to the list, while chloramphenicol was replaced with florfenicol. The E-test method (AB Biodisk, Solna, Sweden) was used until 2004 and was replaced by the broth microdilution method (Sensititre, Trek Diagnostics, Westlake, OH) in 2005.

The breakpoints for determining resistance levels followed the standards established by the Clinical and Laboratory Standards Institute (CLSI) until 2011. In 2012, NARMS adopted the epidemiological cut-off (ECOFF) standards established by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for the interpretation of *Campylobacter*. This change aims to enhance the sensitivity for detecting emerging resistance among *Campylobacter* as well as to standardize the interpretive criteria, so a global surveillance can be conducted. The following sections provide a review of studies that used NARMS data to investigate antimicrobial resistance in *Campylobacter* in the U.S. The ECOFF standards were then applied to the MIC data reported by NARMS from 1997 to 2012 to elucidate trends and pinpoint the emergence of antimicrobial resistance among *Campylobacter* in the U.S.

Antimicrobial resistance in Campylobacter associated with human infections

Before NARMS was established, the CDC monitored antimicrobial resistance in *Salmonella, Shigella,* and *Campylobacter* using periodic surveys of isolates from a panel of sentinel counties. For *Campylobacter*, a sentinel county survey was conducted in 1989-1990, for which 19 randomly chosen counties in all geographic regions of the U.S. participated.^{61, 62} Resistance to tetracycline was observed in 42% of the isolates among a total of 295 *Campylobacter* isolates tested. Two *C. jejuni* isolates were resistant to nalidixic acid (MIC \geq 32 µg/mL), but susceptible to ciprofloxacin (MIC=0.5 µg/mL), whereas resistance to erythromycin and azithromycin was observed in 3% and 2% of the total, respectively. Resistance to clindamycin was also observed in 2%. None of the isolates tested were resistant to chloramphenicol or gentamicin. None of the cases with

Campylobacter isolates resistant to nalidixic acid had a history of foreign travel or treatment with a quinolone or fluoroquinolone in the month before illness.

Concerns regarding the emergence of quinolone-resistant *C. jejuni* in the U.S. were not raised until 1999 following reports from a Minnesota study conducted in 1992 to 1998.²⁹ The testing of 4,953 *Campylobacter* isolates, recovered from patients and submitted to the Minnesota Department of Health, identified an increased proportion of quinolone resistant *C. jejuni* isolates from 1.3% in 1992 to 10.2% in 1998 (*p*<0.001). A significant association was also identified between foreign travel and quinolone-resistant infections (OR=16.0, 95% CI=7.3-38.8, *p*<0.001). However, the number of domestically-acquired quinolone-resistant *C. jejuni* infections had also increased from 0.8% in 1996 to 3.0% in 1998 (*p*=0.002). Consequently, it was suggested that the increase, particularly among the domestic cases, was largely due to the acquisition of resistant strains from poultry since a high frequency of ciprofloxacin-resistant C. jejuni were recovered from meats obtained from retail markets. Further characterization of the resistant strains isolated from domestic cases and from retail chicken products identified similar molecular subtypes by PCR-RFLP among isolates from both sources. The use of fluoroquinolones in poultry began in 1995 in the U.S., which was close to the start of the study period, while the quinolones and ciprofloxacin (a fluoroquinolone) were approved for use in human medicine in the mid-1960s and 1986, respectively.⁶¹ Thus, acknowledging the temporal association and previous reports from other countries on the association between fluoroquinolone use in poultry and the emergence of resistance among human isolates,^{27,63} Smith et al.²⁹ concluded that the use of fluoroquinolones in poultry in Minnesota has created a reservoir

of fluoroquinolone-resistant *C. jejuni* that were readily transmissible to humans. This conclusion was supported by a subsequent study that used NARMS data in 1997-2001.61 Upon testing 1,553 *Campylobacter* isolates collected from nine participating sites, the investigators observed an increase in the proportion of ciprofloxacin-resistant *Campylobacter* isolates from 13% in 1997 to 19% in 2001. The increasing trend was more notable since resistance to other antimicrobials including macrolides, which were also commonly used in human medicine, remained low (1.3%) during the same time period. The study also identified foreign travel, especially to Europe, as a risk factor for ciprofloxacin-resistant *C. jejuni* infections. Over half of the ciprofloxacin-resistant infections, however, were domestically acquired and resistance was not associated with use of fluoroquinolones before specimen collection, another important factor associated with the emergence of resistance. Similar to the Minnesota study,²⁹ Gupta et al.⁶¹ demonstrated that 10% of retail chickens were contaminated with ciprofloxacin-resistant *Campylobacter*. In addition, the FDA conducted a quantitative risk assessment in 2002 on the human health impact of fluoroquinolone resistant *Campylobacter* associated with the consumption of chicken.⁶⁴ The authors concluded that fluoroquinolone use in chickens and turkeys results in >10,000 human infections of fluoroquinolone-resistant *Campylobacter* each year. They further proposed fluoroquinolones no longer be used in poultry, and indeed, a new fluoroquinolone, enrofloxacin, was prohibited for use in poultry in 2005 in the U.S. This guideline marked the first time that an antimicrobial was removed from the market because of its importance for the emergence of resistance in human infections.⁶⁵ These studies further suggested the possible association between fluoroquinolone resistant *C*.

jejuni infections and hospitalization status and longer duration of diarrhea, warranting the need to investigate the clinical outcomes of the fluoroquinolone resistant infections.^{29,61,66}

Based on the raw data from NARMS reports, with ECOFFs applied breakpoints, the prevalence of ciprofloxacin-, and naldixic acid-resistant *C. jejuni* showed a steady, increasing trend from 1997 to 2012 (Figure 1.6.). Importantly, the increase was consistent over the years, even after 2005 when the antimicrobials were withdrawn from use in poultry. Additionally, declining resistance rates to azithromycin and erythromycin were observed over time, while resistance to clindamycin, gentamicin, florfenicol, and telithromycin emerged in recent years with an increasing trend. When comparing the proportion of ciprofloxacin-resistant *C. jejuni* isolates over time between human-, chicken-, and retail meat-derived isolates, a similar increasing trend was observed (Figure 1.10.). Although a steep decline in the proportion of resistant isolates from chickens was observed in 2006, the proportion peaked at over 30% in subsequent years. The similar trends observed between the three sources suggest an association between the prevalence of ciprofloxacin-resistant *C. jejuni* in humans, chicken, and chicken products; however, few studies have been conducted to investigate other possible sources including cattle and environmental waters. Additional studies involving other sources will facilitate our understanding of transmission dynamics of antimicrobial resistant *C. jejuni* in the U.S. and may help guide novel prevention strategies.

Unlike the fluoroquinolones, declining trends were observed for resistance to the macrolides, erythromycin and azithromycin, in all three sources (humans, chickens, retail meat) (Figures 1.11., 1.12.). Nonetheless, similar patterns were observed between sources

for both antimicrobials after 2006, especially between chickens and humans. Tetracycline had the highest resistance rate throughout the time period and over different sources (Figure 1.13.), though no apparent trend was observed.

The overall proportion of resistant *C. jejuni* isolates from 2000 to 2012 showed a substantial decrease of pan-susceptible isolates from humans, while multi-drug resistant isolates showed increasing trends (Figure 1.8.). Multi-drug resistance is an even bigger concern for *C. coli*, as more than 50% of the total *C. coli* isolates tested, and 96.1% of the resistant isolates showed resistance to more than one antimicrobial in 2012 (Figure 1.9.). For each antimicrobial, substantially high resistance rates were observed among *C. coli* isolates for all years (Figure 1.7.), except for florfenicol. When excluding the data from 1997 to 2004 in which only a small number of samples were tested (*n*=4~26), a clear increasing trend was observed for ciprofloxacin, nalidixic acid, clindamycin and tetracycline. Furthermore, resistance to azithromycin, erythromycin, gentamicin, and telithromycin was notably higher than the resistance frequency observed in *C. jejuni* isolates.

SUMMARY AND FUTURE DIRECTIONS

These data highlight the importance of *Campylobacter* infections in the U.S. as well as the antimicrobial resistance, with the increasing trends observed for both. However, there are several limitations associated with the use of surveillance data from FoodNet and NARMS to estimate the incidence and trends of infectious diseases. Most importantly, populations under surveillance and the isolates tested for antimicrobial resistance may not be representative of all populations within the U.S., particularly given that geographic variation in incidence has been described. The ten participating sites are distributed throughout the U.S., but they were not randomly selected, and several sites only include a subset of counties. Thus, caution should be used when interpreting the data and extrapolating to the entire nation. One way to overcome this limitation, as conducted by FoodNet, is to use a statistical model that adjusts for site-to-site variation, different sample sizes, and the estimated under reporting rate. However, more importantly, state-wide epidemiologic studies are warranted outside of the ten FoodNet sites to confirm the trends and identify site-specific risk factors that can be used to guide disease prevention efforts. Also, in this review, a notable time gap was observed for epidemiologic studies conducted at FoodNet sites. Utilizing the current resources and accumulated data at FoodNet sites to conduct an epidemiologic study is greatly warranted to understand the increasing incidence and to identify the associated risk factors.

The antimicrobial resistance of *Campylobacter*, especially against fluoroquinolones, in the U.S. is increasing despite the efforts put in to control the prevalence and antimicrobial use in chickens. Further investigations are warranted to find other potential

reservoirs, i.e. cattle and water, to control the emerging resistance and guide the proper preventive measures. Additionally, speciation and molecular studies should be implemented to study the correct transmission and evolution of the resistant *Campylobacter* strains in the U.S. Specifically, a multilocus sequence typing (MLST) system has been applied for molecular typing of *Campylobacter* since 2001, and now is considered the universal method for studying the molecular epidemiology of *Campylobacter*. By using the defined type by MSLT, a sequence type, one can study the evolution and transmission of *Campylobacter*. This kind of molecular data on *Campylobacter* strains circulating in the U.S. will not only help addressing the role of specific strains contributing to the increased resistance trend, but also to understand the pathogenicity of resistant *Campylobacter* and the relation to the clinical outcomes like hospitalization, deaths, and long-term consequences, i.e. GBS and reactive arthritis. APPENDIX
Figure 1.1. Population under surveillance and the incidence rate of



campylobacteriosis: 1997-2014



Figure 1.2. Incidence rates of campylobacteriosis in each site: 2005-2012

• The site-specific incidence rate is available only from 2005 to 2012.



Figure 1.3. Incidence rates of campylobacteriosis by age group: 2005-2012

Figure 1.4. The overall hospitalization rate due to campylobacteriosis in the U.S.



reported by FoodNet: 1997-2014



Figure 1.5. The trend of hospitalization rate by age group: 2008-2012





NARMS by year: 1997-2012

Figure 1.7. Antimicrobial resistance among *C. coli* collected from humans through



NARMS by year: 1997-2012





isolates: 2000-2012





2000-2012

Figure 1.10. % Frequency of resistance to ciprofloxacin among *C. jejuni* from humans, chickens, and retail chicken meats: 1997-2012



Figure 1.11. % Frequency of resistance to erythromycin among *C. jejuni* from humans, chickens, and retail chicken meats: 1997-2012



Figure 1.12. % Frequency of resistance to azithromycin among *C. jejuni* from humans, chickens, and retail chicken meats: 1997-2012



Figure 1.13. % Frequency of resistance to tetracycline among *C. jejuni* from humans, chickens, and retail chicken meats: 1997-2012



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CHAPTER 2

Epidemiology of campylobacteriosis in Michigan: 2004-2013

ABSTRACT

According to the Michigan Disease Surveillance System (MDSS), a total of 7,128 campylobacteriosis cases were reported in Michigan from 2004 to 2013. Although the incidence rate was comparatively lower than what is reported for the nation by the Foodborne Disease Surveillance Network (FoodNet), an increasing trend was observed, specifically in 20-29 years and >50 years age group. A distinct seasonality was observed with a peak in July, and the trend was more prominent in mid age groups, i.e. 10-19 years, 40-59 years, implying that specific behaviors may contribute to the seasonality. Ageadjusted incidence rates at the county level showed a higher incidence of campylobacteriosis in rural areas compared to urban areas. When stratified by age group, individuals between 10 and 19 years of age had a significantly higher risk of campylobacteriosis in rural areas than 10-19 year olds in urban areas. Factors associated with a higher incidence in rural areas were contact with livestock and drinking untreated well water at home. Approximately 12.5% of the total cases had a history of foreign travel and the most frequent destinations were Mexico, India, and China. A significantly higher hospitalization rate, with an increasing trend over time, was observed in the study compared to the report by FoodNet. Cases older than 60 years were more frequently hospitalized than other age groups, and cases without foreign travel history, and rural cases relative to urban cases were more likely to be hospitalized. The overall finding of increasing incidence and hospitalization rate in this study strongly warrants further studies to investigate the risk factors, accounting for the temporal and spatial patterns in the analysis.

INTRODUCTION

Campylobacter, a zoonotic agent, is one of the most widespread infectious agents in the world.¹ This small gram-negative bacteria is not only the leading cause of gastroenteritis in humans, but also can lead to autoimmune conditions like Guillain-Barré syndrome (GBS),² reactive arthritis,³ and chronic conditions like inflammatory bowel diseases (IBD).⁴ The combined estimated burden of disease is considerable, as it is estimated to cost \$1.7 billion in the U.S. alone.⁵

The annual incidence varies between countries, but the numbers of reported cases have been generally increasing in many countries during the last decade.^{1,6–8} In the U.S., *Campylobacter* infection is the second most common bacterial cause for human gastroenteritis, and there was 13% increase of campylobacteriosis in 2012 when compared to 2006-2008.⁹ The increasing trend can be partly due to the improvement of detection methods as well as the surveillance system, but there also may be certain risk factors responsible for the growing incidence.

Consumption and handling of chicken has been identified as the major risk factor worldwide.¹⁰ Also, raw milk and cheese have been frequently associated with *Campylobacter* outbreaks, suggesting cattle as another major source for human infections.¹¹ Furthermore, the bacteria is widespread in the environment, including water and soil, where it can survive up to several months.^{12, 13} Water, especially, has been identified as an important source for *Campylobacter* infections, occasionally associated with outbreaks.^{14, 15} Human to human transmission by the fecal-oral route is also reported,

however, zoonotic or foodborne transmission predominates. With the high prevalence of *Campylobacter* reported throughout the world, foreign travel has emerged as an important risk factor as well.^{16,17}

Another common characteristic of *Campylobacter* infections is the seasonality. A significantly higher incidence of *Campylobacter* has been described in warmer seasons in different countries, as well as from different sources, i.e. animals and water.^{18,19} The reason behind the seasonality is not fully understood, but has been suggested to be the result of multiple factors including longer survival of *Campylobacter* in the environment, increased shedding levels in animal reservoirs,²⁰ and changes in human behavior.²¹ Also, spatial determinants, i.e., urban versus rural settings, have been reported to be associated with *Campylobacter* incidence,^{22, 23} suggesting the importance of assessing environmental factors when conducting a risk factor analysis.

According to the Foodborne Disease Active Surveillance Network (FoodNet), which tracks the trends of incidence for major food borne pathogens in the U.S., the overall incidence of campylobacteriosis has been showing an increasing trend in the last few years, from 12.30 cases per 100,000 in 2009 to 14.22 per 100,000 in 2012. Also, there is a wide range of incidence between different sites (6.95 in Tennessee to 34.33 in California), which has been relatively constant for each site since the surveillance started in 1996. A study reported no significant difference regarding medical care seeking or medical practices between sites. Furthermore, there were no significant differences observed in the frequency of exposure to risk factors for *Campylobacter* infection between sites. Thus, the current understanding is that the geographical differences in *Campylobacter* incidence are

real, and there are specific environmental factors, i.e. climate, prevalence of *Campylobacter* in reservoirs like chicken in the area, contributing to the different *Campylobacter* incidence in each geographic location. Furthermore, an increasing trend of hospitalization rates due to campylobacteriosis has been reported, from 10% in 1997 to 17% in 2014, warranting the need to monitor the clinical outcomes, including long term consequences like GBS, reactive arthritis, and IBD.

Michigan is not included in the FoodNet surveillance, however, campylobacteriosis is a reportable disease in Michigan, of which health care providers and clinical laboratories are required to report to the local health departments upon diagnosis.²⁴ A notification of a case is sent to Michigan Department of Health and Human Services (MDHHS), and the information about the case, including the demographic, clinical, and epidemiological data, is entered into a web-based surveillance system called Michigan Disease Surveillance System (MDSS).²⁵

Campylobacteriosis was the most frequently reported food-borne disease in Michigan in the last decade, 2004-2013, according to MDSS, even surpassing salmonellosis. Acknowledging the impact on public health, we aimed to investigate the incidence and the associated factors of *Campylobacter* in Michigan using the data from MDSS. We hypothesized that the increasing incidence of *Campylobacter* infections in Michigan, and specific factors, i.e. age, sex, season, and history of foreign travel, were associated with the incidence. We also investigated the clinical outcomes using hospitalization status, and reported symptoms. Lastly, we constructed a map of Michigan at the county level to

examine the risk by the resident location, and sought to identify factors associated with higher incidence.

MATERIALS AND METHODS

Study population

The study population for this research included all residents living in Michigan from January 2004 to December 2013. According to the Bridged-Race Population Estimates 1990-2013 dataset,²⁶ an annual average of 10.56 million population resided in Michigan for the 10-year-period, and the number declined, from 10,055,315 in 2004 to 9,895,622 in 2013. Based on the classification by National Center for Health Statistics (NCHS) data system,²⁷ ten counties that were classified as large metro areas were defined as urban, and the rest of counties were defined as rural in this study. Annually, an average of 5.5 million and 5.06 million Michigan residents lived in urban and rural counties, respectively.

Case definition

A case was defined as a person with a laboratory-confirmed *Campylobacter* infection, reported to the MDSS with the onset date between 1 January 2004 and 31 December 2013. We included only the cases that had the investigation status reported as completed. The electronic investigation form included demographic (e.g. age, sex, race, residence), clinical (e.g. hospitalization status, symptoms), laboratory (e.g. detection method used, species) and epidemiological data (e.g. history of travel, animal contact, water source at home and high risk food exposure).

Data management

All the data was retrieved and managed in Microsoft Excel. Age and race data was grouped based on the current categorization scheme used by the FoodNet,⁹ while season was categorized based on the onset date: spring (March, April, May), summer (June, July, August), Fall (September, October, November) and winter (December, January, February). Travel was considered positive only when the travel period was within one week prior to the onset of symptoms. When the time period was not specified, the case was counted as a missing for analyses involving travel. Including these cases, the remaining cases with known travel history were considered domestically acquired infections. The travel destinations were categorized into eight world regions, based on the classification used by United Nations population division.²⁸ History of food consumption and animal contact data were systematically collected from 2011, thus only the last three years of data were used for the analysis. Animal contact was defined as positive when there was a report of direct contact with reptiles (e.g. snake, lizards), livestock (e.g. cattle, goats, sheep), birds (e.g. chickens, turkey, ducks, parrots), aquatic pets (e.g. fish, turtle), domestic pets (e.g. dogs, cats) and other animals (e.g. rabbit, horse, parakeet). High risk food exposure was considered positive when the case reported 'Yes' to questions asking if the following food items were consumed: ground meats (e.g. turkey, chicken, beef, pork), chicken, (e.g. prepared at home, frozen, or at a restaurant), unpasteurized milk or cheese. The answer choice of 'typically' was not counted as a positive to reduce the information bias. The water source at home was categorized into well, municipal, bottled and other: other included various combinations of different sources, e.g. well and municipal, municipal and bottled

water. Antibiotic treatment was investigated using the information in the supplementary notes section. For all the variables, if the information was missing in the investigation form, it was managed as missing data, along with the ones that were recorded as 'unknown'.

Data analysis

The crude annual incidence rate was calculated by dividing the number of cases by the estimated population in Michigan for each year. The incidence rate per age group and sex, and race was calculated using the population data from the Bridged-Race population estimates. The annual age-adjusted incidence rate was computed with the standard population based on the U.S. 2010 standard population by the U.S. Census Bureau.²⁹

Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences in the frequencies of campylobacteriosis across age group, sex, and other variables including hospitalization, were examined using χ^2 tests; a *P*<0.05 was considered significant. Further analysis was conducted to investigate the association between demographic characteristics and foreign travel history, where the prevalence ratio was compared between travellers with non-travellers. Multivariate analyses for hospitalization and rural versus urban were performed using logistic regression with any independent variable with a *p* value of <0.2 and other variables considered biologically plausible confounders, i.e. age, sex. The model was built using a forward stepwise method with the requirement for a significance level of <0.1 to remain in the model.

All geographic information system (GIS) maps were generated using ArcMap GIS software (version 10.2; ESRI, Redlands, California) using the data from the National Center

for Health Statistics (NCHS) data system, Bridged-Race population estimates, and the case numbers in this study.

RESULTS

Campylobacter speciation and diagnostics in Michigan

Identification to the species level was reported in 2,585 cases (36%). *C. jejuni* comprised the majority of the species identified (*n*=2,540; 98.3%), followed by *C. coli* (*n*=28; 1.1%), *C. lari* (*n*=9), *C. fetus* (*n*=4) and *C. upsalensis* (*n*=1). Three cases reported the species as '*Campylobacter* not *jejuni*'. The detection method was either not specified or just recorded as 'culture'. Among the ones with further information, there were 27 blood culture cases, one vaginal culture, and 271 enzyme immunoassay cases. Notably, use of enzyme immunoassay as the identification method increased from 0.32% in 2004 to 10.19% in 2013 of total cases.

The incidence trend and demographic distribution

A total of 7,128 laboratory confirmed *Campylobacter* cases were reported to the MDHHS between January 2004 and December 2013. The crude mean annual incidence was 7.08 cases per 100,000 population, ranging between 6.23 and 8.43 per 100,000, as observed in 2005 and in 2013, respectively (Figure 2.1.). The age-adjusted incidence rate was similar to the crude incidence, with the average of 7.22 cases per 100,000. Both crude and age-adjusted incidence rates showed significant differences between years, with a trend toward increasing incidence. Only in 2007, 2009 and 2011 was the incidence lower than in the respective previous year. The trend was more evident when the average annual incidence rates of 2007-2010 (6.76 per 100,000) and 2011-2013 (7.23 per 100,000) were compared to the average rate of 2004-2006 (6.26 per 100,000). In addition, outbreak data

was available for 1,423 cases of the total (19.8%), among which 22 cases (1.5%) were identified to be associated with an outbreak. The highest number of outbreak-associated cases was 12 in 2010 followed by five in 2007.

The average incidence was higher for men at 7.88 per 100,000 than women at 6.54 per 100,000, with an incidence rate ratio of 1.20. This incidence ratio, however, increased to 1.24 when adjusted for age (p<0.001) (Figure 2.2.). The median age among all cases was 41 years old with a range between seven days and 100 years old. The highest incidence rate of campylobacteriosis was reported in children younger than 5 years of age (14.86 per 100,000) when compared to other age groups (6.76 per 100,000; p<0.001)(Figure 2.3.). Boys younger than 1 year, however, showed the highest rate affecting 20.64 cases per 100,000 (Figure 2.4.). The lowest incidence was observed in 5-9 years and 10-19 years (4.89 and 4.48 per 100,000, respectively) and the incidence gradually increased, until it peaked at 70-79 years. The difference between sex was most distinctive in <1 year of age, which showed an incidence rate ratio of 1.61 (p<0.01). When the incidence rate was examined by each age group, an increasing trend was observed, especially in groups of 20-29 year olds and individuals greater than 50 years (Figure 2.5.). The incidence among children <10 years of age declined since 2011.

Among cases (n=6,220) with known race information, Caucasians comprised 85% (n=5,284), while 232 (3.7%) and 118 cases (1.9%) were reported from African Americans and Asians, respectively. The remaining 586 cases (9.4%) were reported from individuals with multiple races or with unclassified race. When divided by the population of each race in Michigan during the time frame, the highest incidence was observed in Caucasians

(6.51/100,000) followed by Asians (4.47/100,000) and African Americans (1.56/100,000). Hispanic ethnicity was known in 4,770 cases (66.4%) and 228 of these cases (4.8%) identified themselves as Hispanic or Latino.

Temporal distribution

A marked seasonality was observed, with a distinct peak in July (Figure 2.6.). The trend was observed continuously across all years, though a greater number of cases were reported in July of 2008 and 2013 (Figure 2.7.). When stratified by age group, a similar trend was observed for all groups; however, 10-59 year olds had a more prominent peak in July, whereas cases <1 year and >80 years of age had the lowest peak (Figure 2.8.). Cases between 1 and 9 years occurred more frequently in June than in July.

Characteristics by foreign travel status

Travel status was known for 6,616 cases of the total (92.1%), among which 12.5% (n=825) reported a history of foreign travel within one week prior to onset of symptoms. There was no apparent trend for either the frequency or the proportion of foreign travel cases reported over the years. The frequency of cases without foreign travel history (domestic cases) showed a distinct seasonality (Figure 2.9.), which was very similar to what was observed for the total cases. Indeed, domestic cases were more likely to be reported in the summer, specifically in the months of June and July (OR=1.40-1.51, p<0.01). On the contrary, foreign travel cases were more likely to be reported in winter, specifically in January and February (OR=1.78-1.81, p<0.0001). Cases between 10 and 59 years of age had a significantly higher likelihood to have history of foreign travel (OR=2.37, 95%)

CI=1.98-2.82, *p*<0.0001) then other age groups combined. In addition, males were significantly more likely to report a history of foreign travel than females (OR=1.29, 95% CI=1.11-1.50, *p*<0.001). An association with foreign travel was also identified for Asians and cases of Hispanic or Latino ethnicity. Specifically, the prevalence ratio by race was 4.73 for Asians traveling abroad versus domestically (Table 2.1.).

Overall, by region, Asia was the most frequent destination (29.2%) of the cases with foreign travel history, followed by Europe (21.7%) and Central America (18.3%) (Table 2.2.). By country, 13 countries comprised more than a half of total destinations. Mexico was the most frequently visited country (14.4%) followed by India (7.4%) and Canada (6.4%). Within Europe, France and England were the most visited countries (25.7%), while Peru (51.6%) and Dominican Republic (47.3%) was the most frequently visited countries in South America and Caribbean, respectively.

Geographical distribution

The incidence rate of campylobacteriosis varied considerably among the 83 counties in Michigan (Figure 2.11.). Based on the classification by the National Center for Health Statistics, ten counties in Michigan were classified as large metropolitan areas, which were further defined as urban areas in this study; the 73 remaining counties were defined as rural areas (Figure 2.10.). By this definition, the incidence in urban areas was 6.18 per 100,000 population, while it was 7.47 per 100,000 population in rural areas (p<0.05). Furthermore, when adjusting for age, the ten counties with the highest incidence rates were all in rural areas (Figure 2.12.). To investigate the possibility this urban/rural

difference was due to environmental factors specific to rural areas, we omitted cases with foreign travel history (*n*=825) as well as cases for whom travel information was missing (*n*=566). In this reduced dataset of 5,791 cases, a similar trend was observed for agespecific incidence rates between cases from rural and urban areas; however, the incidence from rural areas was higher for all age groups (Figure 2.13.). Especially, rural cases between 10 and 19 years of age had a higher risk of *Campylobacter* infections than counterparts of urban cases; the incidence rate ratio (IRR) was 1.81. Similarly, rural cases between 20 and 29 years (IRR=1.48), and > 80 years of age (IRR=1.35) were more likely to have *Campylobacter* infections than urban cases in the respective age groups. A higher number of cases was reported in the summer months in both areas, however, more cases were reported in July in rural areas versus urban (Figure 2.14.). Univariate and multivariate analyses were conducted using the epidemiological data to identify additional factors associated with the higher incidence observed in rural areas (Table 2.3.). Univariate analyses showed that contact with animals, i.e. livestock (OR=3.15, 95% CI=2.32-4.30, *p*<0.0001), birds and poultry (OR=1.73, 95% CI=1.33-2.24, *p*<0.0001), and domestic pets (OR=1.8, 95% CI=1.48-2.20, *p*<0.0001), were significantly more frequent among rural cases than urban cases. Also, more cases from rural areas had exposure to raw milk (OR=3.19, 95% CI=1.72-5.92, *p*=0.0001), ground meats (OR=1.44, 95% CI=1.15-1.79, *p*=0.0013), and frozen chicken (OR=1.34, 95% CI=1.01-1.68, *p*=0.01). Additionally, a significantly greater number of rural cases had well water as their primary water source at home compared to the urban cases (OR=7.64, 95% CI=5.94-9.82, *p*<0.0001). Multivariate logistic regression controlling for age and gender identified four risk factors independently associated with *Campylobacter* infection in rural areas: contact with livestock (OR=1.66, 95% CI=1.08-2.55,
p=0.02), consumption of frozen chicken (OR=1.38, 95% CI=1.05-1.82, *p*=0.02), ground meats (OR=1.39, 95% CI=1.08-1.78, *p*=0.009), and well water at home (OR=6.74, 95% CI=4.93-9.20, *p*<0.0001).

Clinical outcomes

The most commonly reported symptom was diarrhea, which was reported in 82.7% of the total cases with the symptom information (n=6,890) (Table 2.5.). Bloody diarrhea was reported in 28.2% (of total n=6,887), and it was more frequently reported from children younger than 5 years old (OR=2.24, 95% CI=1.93-2.60, p<0.0001) compared to other age groups. On the other hand, abdominal pain, chills, fever, fatigue and headache was more frequently reported in 10 – 59 years of age. Based on the notes in the investigation form, there were four cases who reported certain neurologic symptoms, i.e. numbness, tingling in the extremities, upon the follow up investigation, and one of these cases was diagnosed with GBS.

Approximately 25.3% (1,729/6,833) of the total cases with the information were reported to have been hospitalized for *Campylobacter* infections. Overall, the hospitalization rate increased from 23.3% in 2004 to 29.5% in 2013. Average hospitalized days was 3.48 days (*n*=1,592), ranging from 1 to 64 days. Cases older than 60 years old had significantly higher likelihood to be hospitalized than other age groups (OR=2.24, 95% CI=1.98-2.54, *p*<0.0001). Based on the prior associations, we also sought to determine whether there were differences in clinical outcomes among cases in rural versus urban areas and after stratifying by travel history (Table 2.4). Domestic cases without foreign

travel history were more likely to be hospitalized (OR=2.52, 95% CI=2.03-3.12, p<0.0001) compared to the cases with foreign travel history, as well as cases from urban areas versus rural areas (OR=1.16, 95% CI=1.05-1.30, p=0.006). Multivariate analyses showed that all three were independently associated with hospitalization. Nineteen cases died after *Campylobacter* infection was reported (case fatality rate=0.27) and all of these cases were 50 years old or older.

Antibiotic treatment

The information of antibiotic treatment was available in 2,736 cases (38.1%) of the total. Among these, 180 cases (6.6%) reported that they were not prescribed of any antibiotic, while 677 cases (24.7%) did not remember which antibiotic they were prescribed. Ciprofloxacin was the most frequently prescribed antimicrobial (*n*=866; 31.7%), and was occasionally prescribed with other classes of antimicrobials (*n*=197; 22.8%) (e.g. flagyl, azithromycin). Other quinolone class antimicrobial, including levofloxacin, was prescribed in 49 cases. Azithromycin was prescribed in 624 cases (22.8%), most of the times by itself, but in 53cases was prescribed with ciprofloxacin, flagyl, or rifaximin, and sulfamethoxazole/trimethoprim. Erythromycin was prescribed in 182 cases (6.7%) and another macrolide, clarithromycin, was reported in 15 cases. Metronidazole was prescribed in 247 cases (9.0%), and in 137 cases it was given with ciprofloxacin. Other antimicrobials prescribed to the cases included amoxicillin, ampicillin, clindamycin, cefalexin, gentamicin, and vancomycin. Also, doxycycline and tetracycline was prescribed in 30 and 12 cases, respectively.

DISCUSSION

An average annual age-adjusted incidence rate of 7.29 cases per 100,000 was reported for campylobacteriosis in Michigan from 2004 to 2013. This was significantly lower than the incidence rate of 14.22 per 100,000 reported for the nation by the FoodNet in 2012.9 However, it is notable that MDSS is a passive surveillance system, while the FoodNet conducts an active surveillance. The incidence we observed in this study may be a small fraction of the total campylobacteriosis cases occurring in Michigan as not all cases will seek medical attention or all the cases are submitted for *Campylobacter* testing.^{30, 31} Still, assuming the under-reported proportion has not changed over the years, a gradual increasing trend of *Campylobacter* in Michigan was observed in this study. It is possible that the increase is partly due to the change in the interest and testing methods at the clinical laboratories in Michigan. In fact, an increased proportion of cases was diagnosed with an enzyme immunoassay over the years, but it is hard to estimate the impact on the reported incidence. It has been demonstrated that a wide range of practices are conducted in the clinical labs in the U.S. regarding the collection, processing, and isolation of *Campylobacter*,³² which strongly suggests a need for the unified methodology guideline. Only when stable diagnostic practices are in place, can trends in incidence be accurately discerned. Yet, when the incidence rate was stratified by age groups, a clear increasing trend was observed in the 20-29 and >50 year age groups (Figure 2.4.), implicating that the increasing trend may be driven by age-specific risk factors. Significantly higher incidence rates were observed in 2008, 2010, and 2013, and this was mainly due to significantly higher numbers of cases reported in the summer months of these years (Figure 2.7.).

Seasonality of *Campylobacter* has been reported in the U.S., as well as many different countries, e.g. Germany⁷ and England.³³ In the U.S., the incidence peaks between June – August,⁹ and a link between increased incidence and high humidity and temperature has been documented.³⁴ Also, an association between the river temperature in the warmer season and *Campylobacter* survival was found. It is speculated that increased temperature can enhance pathogen survival and proliferation, potentially increasing the load in animal reservoirs.^{13, 20} Also vectors, like flies, can contribute to the increased transmission between animal reservoirs, as well as to humans, when they fly into houses with increased ventilation airflow during warmer weather. Indeed, flies have been implicated as an important vectors of infection for poultry flocks.^{35,36} In addition, people participate in more recreational activities outside during summer months, including barbecuing, camping, fishing, and swimming, greatly enhancing the risk of exposure to the pathogen.²¹ More prominent peaks in July observed among 10-59 years compared to other age groups, especially <1 year and >80 years (Figure 2.8.) support that the behavioral factors contributed significantly to the observed seasonality of campylobacteriosis. Furthermore, when the travel-associated cases were removed, domestic cases showed a marked seasonality in summer months, confirming the factor or factors driving the seasonality of the total cases is most likely a domestic, environmental determinant.

In this study, an increased risk of *Campylobacter* infections in rural areas was observed, which was confirmed by statistical analysis as well as by visualization using GIS mapping. Several ecological studies have been conducted to address the environmental factors contributing to the geographical variation of *Campylobacter* incidence in rural

versus urban settings. While the main transmission route in urban areas is thought to be via consumption of contaminated food products, in rural areas environmental exposure, i.e. direct contact with farm animals,^{37, 38} swimming in the lakes and rivers,³⁹ or drinking untreated water,⁴⁰ is thought to play a larger role in the transmission. In this study, we found that cases from urban areas were more likely to have traveled abroad (OR=1.67, 95% CI=1.44-1.94, *p*<0.0001). Using cases that were acquired domestically, we compared between cases from rural areas versus urban areas to identify the associated factors. The analysis showed that contact with livestock, and drinking well water at home was significantly associated with *Campylobacter* infections in rural areas, regardless of the age group or sex. When age-specific risk was calculated between urban and rural, 10-19 years in rural areas had higher risk for *Campylobacter* (IRR=1.81) compared to the counterparts in urban areas, and also had significantly higher odds of having contact with livestock than other age groups in rural areas (OR=3.2 95% CI=2.0-5.0, p<0.0001). A similar age-specific risk was reported in a study that was conducted in Michigan in 1990s, which reported a higher *Campylobacter* incidence in the counties with higher poultry density.⁴¹ In the study, they reported even stronger associations in young adults and children, suggesting that occupational exposure as well as indirect or environmental exposures is significant. Although we did not observe a clear correlation between the animal density (cattle, poultry) and the incidence rate at the county level by GIS mapping (data not shown), further investigation on the association is warranted using different statistical models, i.e. loglinear model, Poisson regression model. It has been documented that private wells are more vulnerable to contamination, which can happen through sewage overflows, farm runoff and also sewage systems that are not working properly.¹³ When wells are contaminated

with *Campylobacter*, the bacteria can survive up to several months, posing a high risk of transmission to humans.

Previous studies using the FoodNet data reported 18.0-18.9% of the total *Campylobacter* cases to be travel-associated.^{16,17} In this study, 12.5% of the cases were considered to be acquired abroad, showing a significantly lower proportion. When the demographics of travel-associated cases were compared to one of the studies,¹⁶ the median age was higher in Michigan (41 years versus 33.1 years), although a similar age group, 20-59 years was most affected in both studies. By race, a very similar finding was reported: Cases of Asian race were more likely to be travelers than non-travelers, while the opposite was observed among African American. In this study, only 8.7% of cases among African Americans were travel-associated, compared to 39.4% of Asian cases. This could partly explain the low overall incidence rate observed in African American in this study. Furthermore, as previously observed, travel destinations were strongly related to racial and ethnic background: among Asian travelers in this study, 53.5% traveled to Asia, while 41.2% of African American traveled to Africa. This could be due to these cases being immigrants or having friends or family in respective countries, but without the information on the country of origin for cases or the nature of the travel, the association cannot be determined. The FoodNet study further analyzed the risk per the destination regions for *Campylobacter* infections, by using the data of the U.S. residents traveling by air to the destinations in the same time frame.¹⁶ The result showed the highest risk in Africa (35.9 per 100,000), followed by South America (26.4 per 100,000), Central America (17.6 per 100,000), and Asia (15.2 per 100,000). Such detailed data was not available in this study,

but a similar trend was observed for the frequency per destination. When we compared the hospitalization rates between travelers and non-travelers, a significantly higher odds of hospitalization was observed in non-travelers. This is also a consistent finding from the previous studies, which is speculated to be due to the "healthy traveler effect".⁴² Additionally, cases from urban areas were more frequently hospitalized than those from rural areas.

Foreign travel has not been only associated with increased risk of *Campylobacter* infections, but also with the increased antimicrobial resistance, as 60% of travel-associated cases reportedly had a fluoroquinolone-resistant *Campylobacter* isolates previously.¹⁷ Fluoroquinolones and macrolides are the drugs of choice for treating severe cases of *Campylobacter* infections in humans. With the global concern over the emerging resistance to these antimicrobials, a system for monitoring these imported cases is needed, upon which information a proper treatment choice can be made.

Currently, MDSS investigation form does not include the information on antimicrobial treatment. However, 38.1% of the total cases, 20.4-43.4% of cases in each year, had the information in the supplementary note section. Only 6.6% of these cases were not treated with any antimicrobial, while the remainder received at least one antimicrobial for campylobacteriosis: more than a third (35.8%) received fluoroquinolones, while another third (32.2%) was treated with macrolides. Metronidazole was given in 9.7% of the cases and 59% were treated with more than one class of antimicrobials. It was not quantified, however, many of the cases were given ciprofloxacin, a flouoroquinolone, and metronidazole as an empirical treatment for diarrheal disease, then were switched to

macrolides, azithromycin or erythromycin, when *Campylobacter* was confirmed. Interestingly, there were 7 cases treated with vancomycin, to which *Campylobacter* has natural resistance, and 42 cases treated with tetracyclines, to which *Campylobacter* has a high rate of acquired resistance.

Many studies were conducted to investigate the link between the antimicrobial use in food animals, especially chickens, and the emerging resistance in human *Campylobacter* isolates.^{43,44} However, no such effort has been put into studying the impact of the antimicrobial use in human infections for developing the resistance. This is particularly important because resistance to fluoroquinolones typically involves one point mutation in gyrA, which has shown to occur rapidly in chickens after a single dosage.⁴⁵ With the rising concern over the increasing resistance observed globally, the use of fluoroquinolones for the treatment of campylobacteriosis in humans also should be examined. Although this dataset is missing more than 50% of the total cases, this summary nonetheless strongly suggests the need for a system to constantly monitor antimicrobial use, along with testing a subset of samples for the antimicrobial resistance. Speciation of *Campylobacter* should also be recommended since higher antimicrobial resistance and a higher proportion of multidrug resistance have been observed among *C. coli* than *C. jejuni*.⁴⁶ Moreover, updated treatment guidelines and a concerted education program for healthcare provider in Michigan is warranted.

A significantly higher rate of hospitalization and fatality rate was observed among *Campylobacter* cases in Michigan compared to the national report by the FoodNet. However, the FoodNet defined hospitalization when the patient is admitted to a hospital within 7

days after specimen collection, and death only when it occurred during hospitalization, or within 7 days of specimen collection date. In this study, no such time window was applied, thus possibly over-counting the frequency. Still, the increasing rate of hospitalization is alarming, and warrants a further investigation. Moreover, four cases (0.056%) reported neurologic symptoms after a *Campylobacter* infection, among which one case was diagnosed with GBS based on the supplementary notes. A recent systemic review study reported that 0.07% (95% CI=0.03-0.15%) of the *Campylobacter* cases resulted in GBS.⁴⁷ The study also reported 2.86% and 4.01% of the *Campylobacter* cases developed reactive arthritis and irritable bowel syndrome, respectively. Thus, adding a follow-up information section to the case reporting form, or setting up a system to link such cases will greatly benefit monitoring the long-term consequences and morbidity of campylobacteriosis, upon which an epidemiological study, i.e. case-control study, can be conducted to find the associated risk factors.

Additional limitations to the interpretation of surveillance data can come from the ascertainment bias. There is a difference in medical care seeking behavior derived from demographic and socioeconomic status.³¹ For instance, children younger than 5 years are more likely to seek medical care than adults, causing an oversampling bias. Also, higher socioeconomic status has been shown to be associated with a higher notification rate of campylobacteriosis.⁴⁸ In addition, the access to the health care facilities may vary between geographic locations, i.e. urban versus rural. Nevertheless, the findings in this study can be useful in drawing a big picture of campylobacteriosis in Michigan, from which we can focus

and design a further epidemiologic study to address the risk factors, so target specific preventive measures can be designed.

APPENDIX

Characteristic	Total with known Travel status	Total with Travel- known Travel associated status cases		Non-Travel- Associated cases		Prevalence ratio
	No.	No.	%	No.	%	
Total	6616	825	12.5	5791	87 5	
Sex	0010	020	12.0	0,71	0710	
Male	3520	484	58.7	3036	52.4	1.12
Female	3083	339	41.1	2744	47.4	0.87
Unknown	13	2	0.2	11	0.2	1
Age group (years) ^a		_	0.2		0.1	-
<1	176	11	1.3	165	2.9	0.45
1-4	527	31	3.8	496	8.7	0.44
5-19	885	95	11.6	790	13.9	0.83
20-39	1439	247	30.2	1192	20.9	1.44
40-59	2064	314	38.4	1750	30.7	1.25
60-79	1182	111	13.6	1071	18.8	0.72
≥ 80	238	9	1.1	229	4.0	0.28
Median (years)		41.0		42.0		
Race						
Caucasian	4945	562	68.1	4383	75.7	0.90
African American	195	17	2.1	178	3.1	0.68
Asian	109	43	5.2	66	1.1	4.73
Others	552	68	8.2	484	8.4	0.98
Unknown	815	135	16.4	680	11.7	1.40
Ethnicity						
Hispanic or Latino	199	37	4.5	162	2.8	1.61
Non-Hispanic or	4293	481	58.3	3812	65.8	0.89
Unknown	2124	307	37.2	1817	31.4	1.18

Table 2.1. Demographic characteristics of *Campylobacter* cases in Michigan by the

travel status

Table 2.1. (cont'd)

^a Total number =6511

Region	No (%)	Country	No.
Asia	241 (29.2%)	India	61 (7.4%)
		China	37 (4.5%)
		Israel	28 (3.4%)
		Japan	21 (2.5%)
		Thailand	14 (1.7%)
Europe	179 (21.7%)	France	26 (3.2%)
		England	20 (2.4%)
		Spain	11 (1.3%)
Central America	151 (18.3%)	Mexico	119 (14.4%)
South America	64 (7.8%)	Peru	33 (4.0%)
Caribbean	55 (6.7%)	Dominican Republic	26 (3.2%)
North America	52 (6.3%)	Canada	52 (6.4%)
Africa	50 (6.1%)	South Africa	10 (1.2%)
Oceania	6 (0.7%)		
Multiple regions	27 (3.3%)	-	
Total	825	-	458 (55.5%)

Table 2.2. Destinations of *Campylobacter* cases with foreign travel history

• Cases which traveled to multiple destinations were not counted for individual regions.

• Only the countries that were reported from 10 or more cases are listed in the table.

Risk factors	Rural		Urban		Univariate analysis: rural versus urban		
	Cases	%	Cases	%	OR	95% CIα	<i>p</i> -value
Contact with animals							
Livestock	190/972	19.55	59/825	7.15	3.15	2.32-4.30	< 0.0001
Birds/poultry	192/967	19.86	103/822	12.53	1.73	1.33-2.24	< 0.0001
Domestic pets	732/989	74.01	506/826	61.26	1.8	1.48-2.20	< 0.0001
Reptile	47/963	4.88	28/822	3.41	1.46	0.90-2.34	0.12
Aquatic pets	94/957	9.82	62/819	7.57	1.33	0.95-1.86	0.0946
Other	90/924	9.74	52/819	6.35	1.59	1.12-2.27	0.0098
Food consumption							
Food consumption							
Chicken prepared at	475/859	55.3	387/693	55.84	0.98	8 0.80-1.20	0.8292
Frozen chicken	256/862	29.7	167/697	23.96	1.34	1.01-1.68	0.0113
Outside chicken	, 249/885	28.14	, 223/718	31.06	0.87	0.70-1.08	0.2017
Ground meats	507/752	67.42	371/628	59.08	1.44	1.15-1.79	0.0013
Raw milk	49/973	5.04	13/782	1.64	3.19	1.72-5.92	0.0001
Water source at home							
Well	482/1001	48.15	90/830	10.84	7.64	5.94-9.82	< 0.0001
	Multivariate analysis adjusted for age and sex:						

Table 2.3. Univariate and multivariate analyses of risk factors in rural and urban areas

Risk factors	rural versus urban			
	OR	95% CI ^β	<i>p</i> -value	
Livestock	1.66	1.08-2.55	0.0207	
Domestic pets	1.22	0.95-1.57	0.1256	

Table 2.3. (cont'd)

Frozen chicken Ground meats	1.38 1.39	1.05-1.82 1.08-1.78	0.0205 0.009
Raw milk	1.82	0.88-3.74	0.1053
Well	6.74	4.93-9.20	< 0.0001

• Only the cases with the onset date in 2011 – 2013 are included in the analyses.

 $^{\alpha}95\%$ confidence interval for odds ratio

^βWald Confidence interval

Characteristic	Total cases	No (%) hospitalized	OR (95% CI α)	<i>p</i> - value*	
Age groups					
< 10	1139	180 (15.8%))		
10-59	4249	994 (23.4%))		
≥ 60	1441	555 (38.5%)) 2.25 (1.98-2.54)	< 0.0001	
Gender					
Male	3643	892 (24.5%)) 0.91 (0.81-1.01)	0.08	
Female	3176	836 (26.3%))		
Foreign travel					
Yes	813	104 (12.8%))		
No	5683	1533 (27.0%)) 2.52 (2.03-3.12)	< 0.0001	
Season					
Spring	1249	304 (24.3%))		
Summer	2882	749 (26.0%)) 1.06 (0.95-1.19)	0.24	
Fall	1713	448 (26.2%))		
Winter	989	230 (13.3%))		
Residence					
Urban	3261	875 (26.8%)) 1.16 (1.05-1.30)	0.0057	
Rural	3570	845 (23.9%)			
		Multi	variate analysis		
Characteristi	IC	OR 95% CI ^β		<i>p</i> -value	
Age ≥ 60		1.25	1.03-1.51	0.02	
Domestic		2.60	2.08-3.23 <(0.0001	
Urban		1.21	1.22-1.37 (0.001	

Table 2.4. Characteristics of cases with hospitalization status

 $^{\alpha}95\%$ confidence interval for odds ratio

 $^{\beta}$ Wald Confidence interval

Clinical symptoms	Total cases $^{\alpha}$	No. of cases(%)
Diarrhea	6890	5699 (82.7%)
Bloody diarrhea	6887	1945 (28.2%)
Nausea	6889	2839 (41.2%)
Vomiting	6888	1802 (26.2%)
Abdominal pain	6890	4588 (66.6%)
Fatigue	6889	2984 (43.3%)
Headache	6888	1988 (28.9%)
Chills	6889	2730 (39.6%)
Body ache	6888	2059 (29.9%)
Fever	6060	3789 (62.5%)

 Table 2.5. Clinical symptoms of campylobacteriosis in Michigan: 2004-2013

 $^{\alpha}$ Total case with known symptom information



Figure 2.1. Incidence rate of Campylobacteriosis reported in Michigan: 2004-2013



Figure 2.2. Average incidence rate by sex



Figure 2.3. Average annual incidence rate by age group

Figure 2.4. Average annual age- and sex- specific incidence rates of *Campylobacter*



infections in Michigan: 2004-2013



Figure 2.5. Age-specific incidence rates by year: 2004-2013

Figure 2.6. Seasonality of *Campylobacter* cases reported in Michigan by the total



number of Campylobacter cases reported by month, 2004-2013

Figure 2.7. Seasonality of *Campylobacter* cases reported in Michigan by the number

of Campylobacter cases reported by month for each year: 2004-2013





Figure 2.8. Seasonality of *Campylobacter* cases reported in Michigan by the total number of *Campylobacter* cases reported in each month by age group, 2004-2013



Figure 2.9. Seasonality of *Campylobacter* cases by foreign travel status

Figure 2.10. Classification of urban and rural counties



Figure 2.11. GIS map showing crude incidence (cases per 100,000) of *Campylobacter* reported in Michigan by county, 2004-2013



Figure 2.12. GIS map showing age-adjusted incidence (cases per 100,000) of *Campylobacter* reported in Michigan by county, 2004-2013





Figure 2.13. Average age-specific incidence rates by geography: urban versus rural



Figure 2.14. Seasonality of *Campylobacter* cases by geography: urban versus rural

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CHAPTER 3

Antimicrobial susceptibility profiles of human *Campylobacter jejuni* isolates

in Michigan and the association with phylogenetic lineage and disease severity

ABSTRACT

Campylobacter jejuni is a zoonotic pathogen and the most common bacterial cause of human gastroenteritis worldwide. With the increase of antibiotic resistance to fluoroquinolones and macrolides, the drugs of choice for treatment, the CDC recently classified the pathogen as a 'serious' antimicrobial resistant agent. Based on the data from Michigan Disease Surveillance System, Campylobacter was the most commonly reported food-borne pathogen in the last decade. Here, we characterized 94 C. jejuni isolates collected from patients at four Michigan hospitals in 2011 and 2012 to determine the frequency of resistance, and the association with phylogenetic lineage and disease severity. We observed a similar prevalence of fluoroquinolone (19.1%) and macrolides (2.1%) resistance in *C. jejuni* isolates from Michigan as what has been reported for the nation. However, high numbers of the fluoroquinolone resistant *C. jejuni* infections were recovered from patients with a history of foreign travel. A significantly higher prevalence of tetracycline resistant C. jejuni was found in Michigan, and the resistance was linked to multilocus sequence type (ST)-982, which was only recovered from livestock and the environment in the U.S. previously. Furthermore, we found that tetracycline resistant C. *jejuni* were associated with livestock contact (Fisher's *p*<0.05; OR=infinity). These outcomes spur the need to investigate antimicrobial resistance frequencies and molecular epidemiology of *C. jejuni* from livestock and the farm environment to understand the ecology of antimicrobial resistant *C. jejuni* transmission in Michigan, and to further guide mitigation strategies to reduce the prevalence of antimicrobial resistant *C. jejuni* in the area.

INTRODUCTION

Campylobacter spp. are gram negative bacteria responsible for the greatest number of cases of bacterial gastroenteritis worldwide.¹ It is estimated that 1.3 million *Campylobacter* infections occur every year in the U.S., resulting in 13,000 hospitalizations and 120 deaths.² Furthermore, recent studies have demonstrated an association between campylobacteriosis and autoimmune diseases such as Guillain Barré syndrome,³ reactive arthritis,⁴ and irritable bowel syndrome.⁵ According to the CDC, about 89% of human *Campylobacter* isolates found in the U.S. represent C. jejuni, followed by C. coli (8%) and C. upsalensis (2%).6 Campylobacter *spp.* are broad host range pathogens that can colonize the intestinal tracts of chickens, turkeys, pigs and ruminants without causing signs^{7, 8} and survive in water and soil for a long period of time, up to several months.^{9,10} The consumption of contaminated poultry is the major source of sporadic human *Campylobacter* infections,¹¹ while approximately 66% of *Campylobacter* outbreaks are attributed to dairy products, mostly raw milk or cheese.¹² The transmission route is usually through food or contact with contaminated water, but direct transmission from animal sources has also been reported including from household pets such as dogs and cats.^{12, 13}

The most common clinical presentation of campylobacteriosis is self-limiting gastroenteritis with vomiting, cramping, and diarrhea, which lasts for 7 to 10 days in most cases. Many individuals develop more severe and prolonged infections, some with extraintestinal spread of the bacterium, which can lead to meningitis and infections of other organs. In these cases and in infants, geriatric patients, and immunosuppressed patients, treatment with antibiotics is necessary.¹⁴ Ciprofloxacin, a fluoroquinolone that

inhibits DNA synthesis by targeting *gyrA*, and macrolides such as azithromycin and erythromycin, that hinder bacterial protein biosynthesis by targeting 23s *rRNA*, have been recommended as the first line antimicrobials for treatment of campylobacteriosis. Yet, recent isolates found in the U.S. and other countries have shown an increasing resistance to these antimicrobials.^{15,16} In the U.S., the proportion of *Campylobacter* isolates resistant to fluoroquinolones has increased from 14.2% in 1998 to 25.3% in 2012.¹⁷ The increasing resistance, especially for ciprofloxacin, was suggested to be related to the use of enrofloxacin, a fluoroquinolone that is commonly used in poultry.^{16,17} The link between the use of antimicrobials in food animals and the emergence of resistance in human isolates has been observed and documented around the world,¹⁸ warranting the need for continuous monitoring and control of the use of antimicrobials in food animals.

Resistance to fluoroquinolones and macrolides in *Campylobacter* is conferred by point mutations in their target sites, the *gyrA* and *23s rRNA* genes.¹⁹ The C257T point mutation in *gyrA* that yields a Thr-86-IIe amino acid change is the most frequently observed mutation in fluoroquinolone resistant *Campylobacter*. Indeed, prior studies in Finland²⁰ and Hungary²¹ detected the C257T *gyrA* mutation in up to 100% and 98% of fluoroquinolone resistant *C. jejuni* isolates examined, respectively. For macrolides, point mutations A2074C, A2074G, and A2075G in domain V of the 23s *rRNA* gene, have been found to confer a high-level of resistance (Minimal inhibitory concentration (MIC) >128 µg/ml), while A2074T has been shown to confer a low-level of resistance (MIC=8 µg/ml).^{22,23} In addition, the active efflux pump, *cmeABC*, works synergistically with point mutations in these gene targets to simultaneously resist the action of fluoroquinolone, macrolide, tetracycline, beta-lactam, and ketolide antimicrobials.^{19, 24, 25} Tetracycline has

been suggested as an alternative treatment for patients with systemic *Campylobacter* infections with aminoglycosides, like gentamicin,²⁶ but it is rarely used in practice. On the other hand, tetracycline is widely used in food animals like chickens and cattle, for preventive purposes as well as for treatment, e.g. in lambs for abortion.^{27,28} In *Campylobacter*, resistance to tetracycline is conferred by *tet(O)* that has been found widely in isolates recovered from various sources.²⁹ The *tet(O)* encodes a ribosomal protection protein, which induces a conformational change upon binding to the bacterial ribosome, the target site for tetracycline, resulting in the release of the bound tetracycline molecule.³⁰ The *tet(O)* gene can be either chromosomally- or plasmid-encoded (pTet).^{31,32}

There have been conflicting reports on the association between ciprofloxacin resistant *C. jejuni* infections and clinical outcomes. For example, a case-control study conducted in the U.S. reported an association between ciprofloxacin-resistant *Campylobacter* infections and prolonged diarrhea.³³ By contrast, a case-comparison study conducted in the U.K. observed no difference in the severity or duration of acute illness between cases with ciprofloxacin resistant *Campylobacter* infections and ciprofloxacin susceptible infections.³⁴ Thus, additional studies in different human populations are needed to better understand the impact that drug resistant infections have on clinical outcomes. These types of studies will not only enhance understanding of the pathogenicity of antimicrobial resistant *Campylobacter*, but could also impact treatment protocols, especially in the U.S. where empirical treatment with a fluoroquinolone is quite common.

In the U.S., the FoodNet surveillance system was designed to monitor the incidence of common foodborne pathogens, including *Campylobacter*, and the National Antimicrobial

Resistance Monitoring System (NARMS) was designed to examine trends of antimicrobial resistance. Although Michigan is not one of the 10 states included in the FoodNet surveillance system, *Campylobacter* was the most common foodborne pathogen reported through the Michigan Disease Surveillance System (MDSS)³⁵ in the past decade (2004-2013). As a result, this study was undertaken to determine the frequency of antimicrobial resistance in a subset of *C. jejuni* isolates collected in Michigan between 2011 and 2012, and to estimate the genetic diversity of both susceptible and resistant isolates using multilocus sequence typing (MLST). We hypothesized that the frequency of antimicrobial resistance in *C. jejuni* isolates recovered from Michigan patients will be similar to national frequencies reported by NARMS,¹⁷ and that certain risk factors are associated with antimicrobial-resistant *C. jejuni* infections. We also hypothesized that resistance can be linked to specific genotypes and that individuals with resistant infections have more severe or prolonged infections.

MATERIALS AND METHODS

Study population and Campylobacter isolates

A total of 94 *C. jejuni* isolates were characterized for the study. Clinical cases of *Campylobacter* infections are required to be reported to the Michigan Department of Health and Human Services (MDHHS).³⁶ All 94 isolates were from human clinical campylobacteriosis cases identified via a surveillance system set up in collaboration with the MDHHS and four participating hospitals, the University of Michigan Medical Center, Sparrow Health System, Detroit Medical Center, and Spectrum Health Systems, in 2011 and 2012. All protocols were approved by the Institutional Review Boards at Michigan State University (MSU), MDHHS and each of the four participating hospitals. Following receipt at Michigan State University, isolates were cultured on blood plates with cefoperazone, amphotericin B, vancomycin using microaerophilic conditions at 37°C for 48 hours, and multiplex PCR was performed to confirm the species as previously described.³⁷ The isolates were stored in tryptone soy broth with 10% glycerol at -80°C until further testing.

Epidemiological data

Demographic and clinical data was retrieved for each case from the MDSS³⁵ and managed using Microsoft Excel. Three *C. jejuni* isolates were collected from patients whose permanent residences were not Michigan; each case developed campylobacteriosis while traveling in Michigan and thus, epidemiological data was transferred to the respective states (Ohio, New Jersey, and Georgia). These cases were included in the genetic diversity and resistance prevalence estimates, but were excluded from statistical analyses. Twentynine isolates, including the three from out-of-state residents, were also missing all clinical

and epidemiological data and were excluded from further analyses. A history of travel outside of Michigan or the U.S. was considered only when the traveling period was within 1 week prior to the onset of symptoms. The season was classified based on the onset date of symptoms: spring (March, April, May), summer (June, July, August), fall (September, October, November), and winter (December, January, February).

Phenotypic antimicrobial susceptibility profiling

The minimal inhibitory concentrations (MICs) of nine antimicrobials were determined by a standard broth microdilution test following the guidelines of the Clinical and Laboratory Standards Institute (CLSI).³⁸ A 96-well microtiter plate (Sensititre, Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Cleveland, OH) was used for each isolate following the manufacturer's instructions.³⁹ Tested antimicrobials included ciprofloxacin (fluoroquinolone), nalidixic acid, azithromycin (macrolide), erythromycin (macrolide), tetracycline, florfenicol, telithromycin, clindamycin, and gentamicin. *Campylobacter jejuni* ATCC 33560 was used as the quality control strain for every batch, and the breakpoints for each antimicrobial were determined using epidemiologic cut-off values (ECOFFs), following the guidelines of European Committee on Antimicrobial Susceptibility Testing,⁴⁰ per current protocol of the NARMS.¹⁷ When there was bacterial growth at the highest MIC tested for each antibiotic, e.g. 64 µg/mL for tetracycline, the MIC for the isolate was interpreted as the highest MIC tested and greater, i.e. ≥ 64 µg/mL.

Whole genome sequencing

DNA was extracted from all 94 *C. jejuni* isolates using the Wizard genomic DNA purification kit (Promega, Madison, WI) and the concentrations were measured using a

Qubit fluorometer (Life Technologies; Invitrogen, CA). A total of 1µg of DNA per isolate was included in the library, which was prepared with the Nextera XT kit (Illumina, San Diego, CA). Validation of the library size and quantity was performed using a Bioanalyzer (Agilent Technologies, Santa Clara, CA) and KAPA library quantification kit (Kapa Biosystems, Woburn, MA), respectively. The libraries were pooled together for denaturing and sequencing on a Miseq (Illumina) platform for 2x250 reads at the Research Technology Support Facility at MSU. Genomic assemblies were performed *de novo* using Velvet, 1.2.07⁴¹ after trimming with Trimmomatic,⁴² followed by quality checking with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Assemblies were constructed using different kmer values (31, 33, and 35), and the assembly yielding the best N50 value for each isolate was used for downstream analyses. Sequences specific for *gyrA*, 23s *rRNA*, and *tetO* as well as seven MLST loci were extracted from the draft genomes based on reference sequences available on NCBI using the Basic Local Alignment Search Tool (BLAST).⁴³

Multilocus Sequence Typing (MLST)

The MLST profile of each sample was initially determined using the web-based server (www.cbs.dtu.dk/services/MLST) with both the raw reads and assembled contigs following whole genome sequencing. Each gene sequence was also confirmed by Sanger sequencing or PCR-based MLST, as previously described.⁴⁴ Allele, sequence type (ST), and clonal complex (CC) assignments were made using the PubMLST (www.publmlst.org/campylobacter/) database. New alleles (*n*=4) and STs (*n*=6) found in this study were submitted to the database.

In silico analysis of 23s rRNA and gyrA genes

Regions of the 23s *rRNA* and *gyrA* genes, which include the typical point mutation sites associated with resistance to macrolides and fluoroquinolones, respectively, were extracted from the assembled contigs, and aligned by MegAlign (DNAstar, Madison, WI). To confirm the coverage of raw reads on the point mutation sites, the raw sequences for all 94 genomes were mapped using Bowtie2⁴⁵ and viewed them in Tablet⁴⁶ while checking for ambiguity. Additionally, Sanger sequencing was used to confirm the point mutations identified in a subset of samples (n=46) using previously published primers targeting these genes (Table 3.1.).

Determination of the presence and location of tet(0) gene

The presence of *tet(O)* was determined from the genome sequences using the BLAST (<u>http://blast.ncbi.nlm.nih.gov</u>), and the result was confirmed by PCR (Table 3.1.) as previously described.⁴⁷ The location of *tet(O)*, either chromosomal or inserted in a plasmid (pTet) was determined by PCR using previously described primers and conditions.⁴⁸

Data analysis

The frequency map of all campylobacteriosis cases reported in Michigan between 2011 and 2012 (n=1,449) was generated using ArcMap GIS software (version 10.2; ESRI, Redlands, California) using the data extracted from MDSS.³⁵

A Neighbor joining tree (p-distance) with 1,000 bootstrap replications was constructed in MEGA6 based on 7 MLST loci,⁴⁹ to identify evolutionary relationships between strains. Clusters were classified as STs that grouped together with \geq 70% bootstrap support, and parsimonious informative sites were further evaluated for evidence of genetic recombination using Splitstree4.⁵⁰

Statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences in the frequencies of antimicrobial resistance across ST, CC, and other variables including disease presentations and the severity, were examined using a χ^2 and Fisher's exact tests for dichotomous variables, and the student's t-test for continuous variables; a *P*<0.05 was considered significant. Multivariate analysis were performed using logistic regression with any independent variable with a *p* value of <0.2 or as they were considered biologically plausible, i.e. age, sex, for acquiring antimicrobial resistant *C. jejuni* infections. The model was built using a forward stepwise method with the requirement for a significance level of <0.1 to remain in the model.

RESULTS

Description of Campylobacter cases identified in Michigan

Ninety four *C. jejuni* isolates were collected from clinical cases of campylobacteriosis identified at four clinical centers between January 2011 and December 2012. Demographic data and epidemiological data that were used for further statistical analyses are listed in Table 3.2. Among the 94 cases, 55.3% (n=52) were from male, while 39 cases were from female patients (unknown n=3). Children 2 years and younger, and adults older than 50 years comprised about half of the total cases; 22.3% (n=21) and 25.5% (n=24), respectively. Race information was available in 80 cases, and the majority of these cases identified themselves as Caucasian (n=60; 75.0%).

According to MDSS, there were a total of 1,449 laboratory confirmed *Campylobacter* cases in Michigan in 2011 and 2012, and the frequency of reported cases in county level are shown in Figure 3.1. The collection sites, as well as most of the residences of the cases included in this study were derived from the counties with higher frequency of campylobacteriosis reported. Particularly, cases whose resident counties were Wayne, Washtenaw and Oakland, which are the top three counties of highest frequency reported, comprised 61.7% (n=58) of total cases.

Sixty-eight cases had travel history information, among which nine (13.2%) had a history of foreign travel, while 17 (25.0%) had history of domestic travel. Six cases had a history of domestic travel outside Michigan, while 3 cases from other states developed symptoms and were diagnosed with campylobacteriosis when traveling in Michigan. Among 64 cases with a history of animal contact, 38 cases (59.4%) had contact with domestic animals, i.e. dogs and cats. Contact with livestock animals, i.e. cattle, was reported in 7 cases (10.9%), while 6 cases (9.4%) reported contact with birds including poultry, i.e. chickens. Most of the cases with livestock contact (n=6; 85.7%) and poultry contact history (n=5; 83.3%) also reported contacts with dogs and cats.

Antimicrobial resistance profiles of *C. jejuni* isolates and mechanisms of resistance

Thirty isolates (31.9%) were susceptible to all nine antimicrobial agents tested, while 64 isolates (68.1%) were resistant to one or more agents. The highest frequency of resistance was observed for tetracycline (*n*=58 isolates; 61.7%), followed by resistance to both ciprofloxacin and nalidixic acid (n=18 isolates; 19.1%). All C. jejuni isolates resistant to ciprofloxacin and nalidixic acid showed high MICs ($4-32\mu g/mL$, $\geq 64\mu g/mL$). Resistance to florfenicol was only detected in one isolate (1.1%), and all isolates were susceptible to gentamicin. In all, 15 isolates (16%) showed resistance to two or more classes of antibiotics. Thirteen (13.8%) of these were resistant to ciprofloxacin, nalidixic acid, and tetracycline, while two isolates (2.1%) were resistant to both azithromycin and erythromycin as well as ciprofloxacin, nalidixic acid, telithromycin, and clindamycin. Table 3.3. shows the frequency and distribution of MICs over each antibiotic tested, and the distribution of isolates tested over time. There were 34 and 60 C. jejuni isolates collected in 2011 and 2012, respectively. During the study period there was an increase in resistance to all antimicrobials, except for gentamicin and tetracycline, however, the difference was not statistically significant. Furthermore, the distribution patterns of MICs for ciprofloxacin, azithromycin, and tetracycline was similar between 2011 and 2012 (Figure 3.2.).

All 18 *C. jejuni* isolates that were phenotypically resistant to ciprofloxacin had a point mutation at 257 in *gyrA*; 17 isolates had the C257T mutation, while one isolate had double mutations of C257G and A258G, resulting in an amino acid change of Thr-86-Ile and Thr-86-Arg, respectively. Two isolates that were resistant to azithromycin and erythromycin had an A2074T point mutation in their 23*s rRNAs*, and all 58 tetracycline resistant isolates harbored *tet(0)*; 18 (31.0%) were inserted in pTet plasmids.

Epidemiological associations with antimicrobial resistant C. jejuni infections

To identify factors associated with antimicrobial resistant *C. jejuni* infections, we conducted univariate analyses using demographic and epidemiological data in cases with available information (Table 3.4.). Notably, cases reporting a history of foreign travel showed a higher likelihood of ciprofloxacin- and nalidixic acid- resistant (CipNal) *C. jejuni* infections (Fisher's p<0.0001) with the odds ratio of 35.7 (exact 95% CI; 4.37, 312.1). In the nine cases with a foreign travel history, seven were resistant to ciprofloxacin and nalidixic acid. Six of these isolates were also resistant to tetracycline, yielding another significant association between foreign travel and a resistance profile; ciprofloxacin-, nalidixic acid-, tetracycline- resistance (CipNalTet) (Fisher's p<0.0001; OR=35.3). In addition, cases with CipNalTet resistant infections were more frequent in the winter months (December, January, February) compared to other seasons (Fisher's p<0.05; OR=4.63). Among food consumption history, eating chicken prepared at home was found to be a protective factor for CipNal infections (p<0.05; OR=0.086) as well as CipNalTet infections (p<0.01; OR=0.0). Meanwhile, contact with livestock was associated with tetracycline-resistant (Tet) *C. jejuni*

infections (Fisher's p<0.05; OR=infinity). For Tet resistant infections, eating frozen chicken was found to be a protective behavior (p=0.01; OR=0.157).

In order to elucidate the factors associated with antimicrobial resistant *C. jejuni* infections exclusively in Michigan, we conducted the same univariate analysis using data from only the Michigan cases (n=53), excluding the cases missing the travel information (n=26) and those with a travel history in foreign countries (n=9) or out of state (n=6) (Table 3.4.). The result showed that a contact with livestock animals was associated with tetracycline resistant infections, however, the p-value increased to 0.0538 (OR=infinity) due to the smaller sample size. Consumption of frozen chicken was a protective factor for Tet resistant infections (p<0.05; OR=0.22).

Multivariate analysis was conducted to model the risk of acquiring CipNal resistant *C. jejuni* infections in all cases (*n*=94), using the factors with significant associations (*p*<0.2) found in the univariate analyses along with biologically plausible factors, i.e. age, sex (Table 3.5.). The base model included history of foreign travel and season (winter), which were independently associated with CipNal infections even when the age and sex was included in the model. The odds ratio was 40.79 (95% CI=5.34-311.50) and 8.86 (1.04-75.16) for foreign travel history and winter in the final model, respectively. When the same multivariate analysis was performed for CipNalTet resistant *C. jejuni* infections, the same variables, history of foreign travel and season, were left in the final model. These were independently associated with CipNalTet infections with increased odds ratios of OR=54.22 (95% CI=4.1-717.12) and OR=25.3 (95% CI=1.58-405.73) for foreign travel history and winter, respectively. However, there was no association found between foreign travel history and season (winter). Although having chicken prepared at home was a protective

factor in univariate analysis, this variable could not be forced into either of these models, as it significantly reduced the sample size for analysis. Similarly, multivariate analyses for Tet infections in Michigan cases (n=53) was not performed due to the small sample size.

Genetic diversity and phylogenetic structure of C. jejuni

A total of 49 different STs, including six novel STs, were represented among all 94 C. *jejuni* isolates recovered in Michigan (Figure 3.3.). These STs were assigned to 17 clonal complexes (CCs), while 11 STs were singletons. The six new STs were assigned to ST-6749 (CC-353), ST- 6751 (CC-61), ST-6752 (CC-353), ST-6788 (CC-1332), ST-7009 (CC unassigned), and ST-7010 (CC unassigned). The most prevalent STs were ST-982 (*n*=10; 10.6%) and ST-353 (n=9; 9.6%), followed by ST-45 (n=7; 7.4%), ST-50 (n=5; 5.3%) and ST-48 (n=4; 4.3%). Thirty four of the remaining STs had only one isolate assigned to each ST. Because a high frequency (41.2%) of cases reported a history of travel within 1 week prior to developing a *C. jejuni* infection, we stratified the distribution of CCs by travel history and the location (Figure 3.4.). The most prevalent CCs with isolates from the cases with foreign travel history were CC-21 (n=4) and CC-464 (n=2). Two of the three isolates that were assigned to CC-464, which was comprised of only ST-464, were both from cases with foreign travel history, while the other was missing any travel information (Figure 3.4.). Meanwhile, most of the isolates assigned to CC-45, CC-48, and CC-42 had no history of travel outside Michigan.

The number of isolates per each ST and the resistance pattern are shown in Figure 3.4., along with their CC assignment. The MLST-based Neighbor-joining phylogeny for all 94 isolates showed that some STs were closely related, though the bootstrap support was low,

which is likely due to the high diversity and frequent recombination among STs in this isolate population (pairwise homoplasy index (PHI)=0.0). Indeed, an evaluation of the 144 parsimonious informative sites provided evidence of significant recombination among the STs via a Neighbor-net analysis (Figure 3.5.). In order to elucidate the ST distribution and evolutionary relationships of isolates that were restrictively derived from Michigan, we excluded nine and six cases with foreign travel and out of state travel history, respectively, as well as 26 with missing data. A total of 35 different STs, including four novel STs remained to represent 53 cases that had no history of travel (n=42) or travel within Michigan (n=11). Although there was still evidence of recombination between these isolates (Figure 3.6.), the phylogenetic tree with Neighbor-joining method showed enhanced bootstrap support, and five distinct clusters with significant bootstrap support ($\geq 70\%$) were observed (Figure 3.7.).

Association between phylogenetic lineage and epidemiologic data

Multiple epidemiological factors were identified as associated with specific *C. jejuni* genotypes. For example, a history of foreign travel was significantly associated with infections caused by ST-464 (CC-464) isolates (Fisher's p<0.05), while infection with ST-982 was linked to contact with livestock (Fisher's p<0.05). Furthermore, drinking water from a well at home (Fisher's p<0.05), and contact with birds (Fisher's p<0.01) were associated with ST-982, while contact with birds (Fisher's p<0.05) and female gender (Chi-square p<0.05) were associated with CC-21 (n=25). While infection with a CC-257 isolate was associated with domestic travel (Fisher's p<0.01), well water at home (Fisher's p<0.05), and contact with livestock (Fisher's p<0.05), and contact with livestock (Fisher's p<0.05), were associated with CC-21 (n=25). While infection with a CC-257 isolate was associated with domestic travel (Fisher's p<0.01), well water at home (Fisher's p<0.05), and contact with livestock (Fisher's p<0.05), the sample size (n=3) was small and two

individuals were from the same household. Finally, cases older than 50 years of age were more likely to have isolates belonging to CC-45 (n=9) (Fisher's p<0.05).

Among the 53 cases derived only from Michigan, similar associations were observed. Notably, contact with chickens was associated with ST-982 (Fisher's p<0.05), as well as CC-21 (Fisher's p<0.05). Because the phylogenetic clusters were better defined in the isolate population exclusively from Michigan cases (Figure 3.7.), we also analyzed epidemiologic associations with the clusters. The isolates belonging to Cluster IV, which includes ST-982 and CC-21, was significantly associated with contact with chickens and ducks (Fisher's p<0.05). Furthermore, when compared to other clusters, Cluster IV showed a significant association with livestock contact (Fisher's p<0.05).

Association between phylogenetic lineage and antimicrobial resistance

All three isolates that were assigned to ST-464, and CC-464 consequently, had the same resistance profile and were resistant to ciprofloxacin, nalidixic acid and tetracycline. A significant association was observed between ST-464 (CC-464) and resistance to ciprofloxacin, nalidixic acid (Fisher's p<0.01), and ciprofloxacin, nalidixic acid, tetracycline (Fisher's p<0.01). The isolates that were assigned to ST-982 had higher likelihood to be tetracycline resistant (Fisher's p<0.05). There was no other ST or CC that had significant association with a specific resistance profile. For the 53 cases from Michigan, analysis of resistance profile by cluster showed that cluster IV had a significant association with tetracycline resistance (Fisher's p<0.05). Further analysis by ST showed that ST-982 in cluster IV had a significant association with tetracycline resistance (Fisher's p<0.05).

Correlation with severity of disease

For determining the severity of disease, we used reported symptom data from each case as well as the hospitalization status, and the length of hospitalization. Table 3.6. shows the list of variables we used for assessing the severity of disease. About 28% of total cases with information available (n=83) were hospitalized for an average of 3.43 days. The most frequently reported symptom was diarrhea (98.8%), followed by abdominal pain (66.7%) and fever (54.7%). When the frequency of these variables was compared between cases with ciprofloxacin-resistant, and ciprofloxacin-susceptible *C. jejuni* infections, no significant difference was observed (Table 3.6.). Duration of illness, calculated from the onset date and recovery date, was available for analysis in 41 cases (43.6%). There was difference in the mean duration of illness between cases that had ciprofloxacin-resistant (11.78 days) and ciprofloxacin-susceptible C. jejuni infections (8.78 days), but the difference was not statistically significant (student's *t* test p=0.12). The same analysis was performed for tetracycline resistant infections as well as multiple drug resistant infections, but no significant associations were observed between antimicrobial resistant *C. jejuni* infections and disease severity measures. Furthermore, there was no significant association observed between any of genotypes discussed above, i.e. ST, CC, cluster, and the severity of disease.

DISCUSSION

The 2012 NARMS report showed 25.3% and 1.8% resistance to ciprofloxacin and azithromycin, respectively, for human *C. jejuni* isolates (n=1,191) in the U.S. In comparison, our study showed lower resistance rates to ciprofloxacin (19.1%) and higher resistance rates for azithromycin (2.1%). However, none of the differences were statistically significant. Nalidixic acid, a quinolone, is not used for treatment in the U.S., but the resistance is frequently screened because of its close correlation with fluoroquinolone resistance in *Campylobacter spp*. In our study, all ciprofloxacin resistant strains (n=18) also showed high resistance to nalidixic acid (MICs $\geq 64\mu$ g/mL).

Thirty six isolates (38.3%) had at least one point mutation in their *gyrA*, while only 18 of them had non-synonymous point mutations (Table 3.7.). All 18 of these isolates were resistant to both ciprofloxacin and nalidixic acid; 17 isolates had a point mutation of C257T in the *gyrA* resulting in amino acid change of Thr-86-Ileu, while one isolate had a double mutation of C257G and A258G mutation yielding Thr-86-Arg change. Thr-86-Arg has been documented as one of the mutations in fluoroquinolone resistant *C. jejuni* and *C. coli*,⁵¹ however, it has rarely been described in the recent literature. The isolate also showed resistance to 3 other classes of antibiotics. Thus, we speculate that there had been involvement of efflux pump, i.e. cmeABC, conferring the high resistance for ciprofloxacin (MIC=16µg/mL) in the isolate. There was no pattern observed in the type or frequency of the synonymous point mutations associated with the resistance profiles or the minimum inhibition concentration (MIC). However, notable associations were observed between STs and the kind of point mutation. For instance, most isolates of ST-45 and ST-353 showed

T234C and C330T mutations in their gyrA genes, respectively, while none of ST-982 had any synonymous point mutations. In fact, gyrA has been suggested to be a useful genetic marker for investigating the genetic relatedness of *C. jejuni* strains,^{52–54} and our finding supports the results from previous literature. For 23s rRNA gene, only seven isolates had synonymous point mutations which included the multiple drug resistant (MDR) isolates that showed resistance to 4 different classes of antimicrobials, including macrolides (azithromycin, erythromycin), a fluoroquinolone (ciprofloxacin), a lincosamide (clindamycin) and a ketolide (telithromycin). None of the MDR isolates was assigned to a CC, but they were close in the phylogenetic tree (Figure 3.3.) as one isolate was ST-6 and the other was ST-5221. The two isolates further shared very similar profile of synonymous point mutations in the 23s rRNA (Table 3.8.). ST-7010, which was found in the same node in the phylogenetic tree, also had very similar point mutations in 23s *rRNA*, except for A2074T, which is responsible for macrolide resistance. Among point mutations discovered for macrolide resistance, A2074T is known to confer low level resistance. However, in this study both macrolide resistant isolates had A2074T, while showing high resistance for both erythromycin and azithromycin (MIC \geq 64ug/ml). Macrolide resistance is known to cause a fitness burden for *C. jejuni*,⁵⁵ so it is unlikely that there is a clonal spread of macrolide resistant *C. jejuni* in Michigan. However, observing the similarity of point mutation profiles in 23s rRNA (Table 3.7.), and the close genetic relatedness in the phylogentic tree of the three MDR isolates suggests that there may be a *C. jejuni* clone circulating in the area with increased expression of the efflux pump. This finding warrants further investigation on the level of expression of *cmeABC* in these isolates.

A significantly higher tetracycline resistance rate (61.7%) was observed in the study samples when compared to the 2012 NARMS report (47.8%) (Chi-square p<0.01). All of the tetracycline resistant *C. jejuni* isolates (n=58) harbored *tet(0)*, but the prevalence of plasmid-mediated tet(0) was comparatively lower than other studies.³² About 31% (*n*=18) of the tet(0) was shown to be inserted in pTet in our study, while studies in Canada⁴⁷ and Germany³² found 67% and 54% plasmid- mediated the tetracycline resistance, respectively. These differences could be due to the different detection methods used in this study. Previous reports extracted the plasmids from the isolates, then performed a conventional PCR for tet(0),⁴⁷ while we used the whole DNA and targeted both pTet and tet(0) with one primer set.⁴⁸ The pTet plasmid that confers tetracycline resistance, is the plasmid that has been found to have the *tet(O)* insertion in *Campylobacter*.⁵⁶ However, we cannot exclude the possibility of other plasmids being involved in carriage of tet(0), warranting the need to confirm the location of tet(0) in our isolates using whole genome sequences with annotation and mapping. Meanwhile, a significant association between a genotype, ST-982, and tetracycline resistance was observed (p < 0.05; OR=6.75), among which only 2 of 9 tetracycline resistant isolates had plasmid-inserted tet(0). According to the Pubmlst database, ST-982 has only been reported from cattle (n=14), cow's milk (n=4), the farm environment (n=3) and a lamb (n=1) in the U.S. before this study. However, this ST has been frequently reported from human clinical cases in other countries e.g., Canada and U.K. Furthermore, a study that was conducted in the state of Washington⁵⁷ isolated ST-982 from both human and cattle samples, confirming it is not a new ST that just emerged in human population in the U.S., but still showing the significant association with cattle. Indeed, a significant association between contact with livestock, i.e. cattle, and ST-982

(*p*<0.05; OR=13.5), and tetracycline resistance (*p*<0.05; OR=infinity) was observed. Taken together, these findings highly suggest that the high rate of tetracycline resistance observed in human isolates in this study is related to the cattle in the area. Further investigation of genetic diversity and antimicrobial resistance of *C. jejuni* in livestock, i.e. cattle, will help clarify the dynamics of potential tetracycline resistant *C. jejuni* transmission between humans and cattle in Michigan.

Among MDR profiles, a high level of ciprofloxacin-, nalidixic acid-, tetracycline-(CipNalTet) resistance was observed in this study. The combination of fluoroquinolone and tetracycline resistance has been observed in other studies as well.⁵⁸ In our study, CipNalTet resistance was significantly associated with foreign travel to diverse geographic locations. We speculate that the high frequency of the CipNalTet resistance observed in this study is due to the high frequency of fluoroquinolone and tetracycline resistant *C. jejuni*, individually, so the coincidental combination is more likely to occur than with others. CipNalTet resistance was also observed more often in winter months, however, there was no association found between foreign travel history and season by multivariate analysis.

We could not identify valid clusters on the phylogenetic tree with significant bootstrap support from all isolates, due to the number of *C. jejuni* strains that were imported from foreign countries and other states (Figure 3.3.). Nevertheless, the same CCs that were assigned based on the Pubmlst database were more closely related on the tree. CC-21 was the most prevalent CC in this study, accounting for 25 isolates (26.6%). And as shown in Figure 3.3., the isolates came from various regions, including 4 other countries and 2 other states. On the other hand, CC-353 and CC-45, to which was assigned 15 and 9

isolates, respectively, were mostly acquired domestically. CC-464 that contained 3 isolates was comprised of only ST-464. Two of the cases had a history of foreign travel, while the third case was missing all epidemiologic information. While the ST is quite prevalent in European and Asian countries, this is the first time ST-464 is reported in the U.S according to the Pubmlst database. The association found between the ST and history of foreign travel in this study highly suggests that all 3 isolates were acquired abroad.

The association between foreign travel and fluoroquinolone resistant C. jejuni infections has been reported worldwide including in the U.S.,^{59,60} and various countries in Europe.^{61–64} In fact, the observation was made almost immediately after the notion of fluoroquinolone resistance was raised,⁵⁹ and importantly, the travel locations included not only developing countries, but also developed countries. A case-control study⁶⁵ that was conducted using FoodNet surveillance sites in the U.S. during 1998-1999 showed that having a history of foreign travel resulted in higher likelihood of acquiring fluoroquinolone-resistant *Campylobacter* infections (OR=7.6; 95% CI=4.3-13.4), while consumption of poultry outside of the home was the major risk factor for domesticallyacquired fluoroquinolone-resistant infections (OR=10.0, 95% CI=1.3-78). In our study, foreign travel had a higher impact on acquiring fluoroquinolone-resistant *C. jejuni* infections (OR=35.7. CI=5.78-220.38), while there was no risk factor identified for domestically acquired fluoroquinolone-resistant infections, most likely due to the small sample size. The literature shows that the reason for high frequency of travel-associated fluoroquinolone resistant *Campylobacter* infections can be due to high prevalence of fluoroquinolone resistance in the destination areas.^{15,59} After the association between the use of fluoroquinolones in poultry and the rising incidence of fluoroquinolone resistance in

humans was confirmed, many countries had raised caution and some banned the use of fluoroquinolones in poultry.⁶⁶ But, both epidemiologically and experimentally, it was shown that fluoroquinolone resistance, once established, can persist in the natural host, i.e. chickens, even after the selective pressure has been removed.^{67,68} The high association between fluoroquinolone-resistant *Campylobacter* infections and travel history in developed countries found in this study may be due to two factors. The first factor is the persistence and transmission of these resistant strains in the area. And second contributing factor could be the weakened immune status of the host. It is more likely that a person on foreign travel is physically stressed due to lack of sleep, exposure to different environmental factors, including water, and may also be more susceptible to the new strains of *Campylobacter* because of the lack of previous exposure to induce immunological memory.

There was no correlation found between the resistance to tested antimicrobials, including ciprofloxacin, and the severity of disease. However, significant associations were observed between age and some of the clinical variables we used in the study to assess the disease severity. One factor to note is the association between young age (0-2 years) and presence of bloody diarrhea (p<0.01, OR=10.45). Interestingly, studies conducted in Hungary²¹ and U.K.⁶⁹ also found the same association. Since these findings are consistent despite different geography and different strains involved, we speculate that the observation can be genuinely attributed to the immaturity of intestinal mucosal immune system in the young infants^{69–71}, rather than a result of ascertainment bias. On the contrary, a higher likelihood of hospitalization (p<0.01; OR=6.18) and longer hospitalization (student's t-test, p<0.01) were observed in *C.jejuni* cases with age above 50. These cases

also had a higher likelihood to report symptoms like body ache (p<0.01, OR=4.54), chills (p<0.01, OR=9.85), fatigue (p<0.05, OR=3.04) and nausea (p<0.05, OR=3.04). When we looked further into the details, we found some of the cases with age over 50 had comorbidities that required hospitalizations and longer stays in hospitals. Concomitant disease and aging would be expected to be a contributing factor to general immune suppression driving enhanced susceptibility in the elderly. These findings suggest that presence of bloody diarrhea and hospitalization status or length of hospitalization might not be complete parameters for evaluating the severity of disease, unless age and underlying diseases are handled as either a confounding or interacting factor in the study design or in the analysis.

Previous studies that reported more severe clinical outcomes in ciprofloxacin resistant *C. jejuni* infections all used the duration of illness, i.e. duration of diarrhea, as the clinical outcome.^{33,59,72} However, the nature of this variable makes it very dependent on whether and when the case was treated with antidiarrheal medication or antimicrobials. Among three previous studies that examined this factor, two that were conducted in the U.S. had the treatment data included in their analyses.^{33,59} One of these studies that was conducted in Minnesota⁵⁹ reported a longer duration of illness in ciprofloxacin-resistant cases (10 days) compared to ciprofloxacin-susceptible cases (7 days, *p*=.03). However, the finding was for cases treated with only ciprofloxacin, so it is not surprsing that the patients infected with ciprofloxacin. The other U.S.-based study was conducted in multistate FoodNet sites and also reported significantly longer duration of diarrhea in ciprofloxacin-resistant infection cases (12 days vs 6 days, *p*=.04). However, the small sample size (ciprofloxacin-

resistant infection n=7), and some contradictory findings in the study itself, i.e. no significant difference in likelihood of hospitalization and missed working days between cases with resistant and susceptible infections, make the validity of their conclusion questionable.⁷³ In our study, only 41 cases (43.6%) had information on duration of illness, and a difference was observed between ciprofloxacin-resistant cases (n=9; 11.78 days) and ciprofloxacin-susceptible cases (n=32; 8.78 days). However, the difference was not statistically significant due to the wide range of duration observed in both cases. Furthermore, only 22 of these cases had information on the antimicrobial treatment, among which six cases had information on prescription date, making it hard to draw a valid conclusion.

In total, antimicrobial treatment information was available for 35 cases (37.2%), while there was no information collected on antidiarrheal medications. Thirteen cases were given ciprofloxacin and 10 cases were given either azithromycin or erythromycin. Six cases did not remember the antibiotics they were prescribed and the remaining cases were prescribed other classes of antibiotics including sulfamethoxazole/trimethoprim, levofloxacin, amoxicillin, ampicillin, and metronidazole.

There were some drawbacks to this study as is commonly found when using surveillance data. The high proportion of missing data and non-uniform measurements, i.e. duration of illness, antimicrobial use, made it difficult to draw a valid conclusion on the research questions. In fact, twenty-nine (30.9%) of 94 total cases were missing all epidemiologic information, including the travel history, hindering a complete analysis. However, antimicrobial resistance patterns and genotype distribution were not significantly different between the cases that were included in the analysis (n=65) versus

the ones that were excluded (n=29). We also acknowledge the geographic limitation of our samples, as the collection of *C. jejuni* isolates from clinical cases came from 4 hospitals in Michigan, which were all located in the big cities (Figure 3.1.). In fact, as shown in Figure 3.6., many of the singletons that did not cluster with other STs (*) were from cases with a travel history in Michigan, e.g. upper peninsula, that were not adjacent to the sample collection sites. However, when we compared the distribution of age, sex, and race between our samples and the cases that were reported to MDSS in 2011-2012 (n=1,449), a very similar distribution was observed (data not shown).

This is a cross-sectional epidemiological study investigating the antimicrobial resistance of *C. jejuni,* the associated factors, and the genetic diversity. We observed a similar prevalence of fluoroquinolone and macrolide resistance in *C. jejuni* isolates from Michigan to what was reported for the nation in 2012. A high number of fluoroquinolone resistant *C. jejuni* infections were acquired from foreign countries, warranting the need to monitor the potential dissemination and evolution of the imported fluoroquinolone resistant *C. jejuni* strains in human population. A significantly higher prevalence of tetracycline resistance was observed in this study compared to the national report, and the resistance was significantly associated with contact with livestock, specifically cattle. A specific genotype, ST-982, was linked to both tetracycline resistance and livestock contact, implying that a tetracycline resistant, pathogenic clone may be circulating in the cattle population in Michigan. This finding warrants a further study to characterize the cattle isolates from Michigan for the antimicrobial resistance and genotypes by MLST. We speculate the data presented in this study reflects well the snap-shot of *C. jejuni* isolates in Michigan, from which important questions can be drawn for future studies.

APPENDIX

 Table 3.1. Primers used for PCR amplification and Sanger sequencing of

Gene	Primer	Sequence	Size (bp)
gyrA ⁷⁴	F-campy gyrA1	5'-TTT TTA GCA AAG ATT CTG AT-3'	
	R-campy gyrA4	5'-CAG TAT AAC GCA TCG CAG CG-3'	368
23s rRNA ⁷⁵	F-campy-23S	5'-AAGAGGATGTATAGGGTGTGACG-3'	
tetO47	R-campy-23S	5'-AACGATTTCCAACCGTTCTG-3"	508
	F-campy tetO	5'-GCGTTTTGTTTATGTGCG-3'	
<i>tet0</i> ⁴⁸	R-campy tetO	5'-ATGGACAACCCGACAGAAG-3"	579
(plasmid)	tetOF1	5'-TAG CCG TAT AGA TAA GGT TCG-3'	
	cpp6-R1	5'-CTG TGC ATA AAA TCA TAG AAT-3'	~3,500

Demographic data	No. of cases	(%)†	Epidemiologic data	No. of cases	(%)†
Sex ^a			Travel ^d		
Male	52	57.1%	No travel	42	61.8%
Female	39	42.9%	Domestic travel	17	25.0%
Age group (years)			Foreign travel	9	13.2%
≤2	21	22.3%	Animal contact ^e		
3-23	25	26.6%	Reptile	0	0%
24-50	24	25.5%	Livestock	7	10.9%
>50	24	25.5%	Birds/poultry	6	9.4%
Race ^b			Domestic	38	59.4%
Caucasian	60	75.0%	Others	5	7.8%
African American	9	11.3%	Food consumption ^f		
Asian	1	1.2%	Ground meats	33	55.0%
Others	10	12.5%	Home prepared		
Residence (county) ^c			chickens	30	50.0%
Clinton	4	4.6%	Frozen chickens	14	23.3%
Ingham	8	9.2%	Restaurant chickens	19	31.7%
Livingston	6	6.9%	Raw sprouts	4	6.7%
Macomb	3	3.4%	Raw milk	4	6.7%
Oakland	10	11.5%	Water at home ^g		
Washtenaw	17	19.5%	Well	12	19.0%
Wayne	31	35.6%	Municipal	42	66.7%
Others	8	9.2%	Bottled	7	11.1%
			Others	2	3.2%

 Table 3.2. Description of cases included in the study

• Counts for sex, age group, race, travel history, and water source at home were mutually exclusive for each category.

Table 3.2. (cont'd)

- Counts for animal contact and food consumption were not mutually exclusive, and were counted repeatedly across categories as they were reported.
- † The percentages are based on the number of cases for which information was available.

^a Unknown cases n=3 (3.2%)

- ^b Unknown cases *n*=14 (14.9%)
- ^c Unknown cases n=7 (7.4%)
- ^d Unknown cases n=26 (27.7%)
- ^e Unknown cases *n*=30 (31.9%)
- ^fUnknown cases *n*=34 (36.2%)
- ^g Unknown cases n=31 (33%)

CLSI*	Antimicrobial	%	Percentage of isolates at the indicated MIC (μ g/mL)†														
Class	agent	2011 (<i>n</i> =34)	2012 (<i>n</i> =60)	Total (<i>n</i> =94)	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Fluoroquinolone Ciprofloxacin	Ciprofloxacin	14.71%	21.67%	19.15%	1.1		42.6	29.8	7.4				1.1	10.6	5.3	2.1	
	r	(5)	(13)	(18)												-	
Quinolone Nalidixic acid	Nalidixic acid	14.71%	21.67%	19.15%									681	117	11		191
	inananie uciu	(5)	(13)	(18)									00.1	11.7	1.1		17.1
Azithromycin Macrolide Erythromycin	Azithromycin	0	3.33%	2.13%	53	27.7	39.4	20.2	5.3								21
	i izitini oniy tini	0	(2)	(2)	5.5												2.1
	Frythromycin	0	3.33%	2.13%		11	11	64	32.9	30.9	20.2	53					21
	Liythioniythi		(2)	(2)		1.1	1.1	0.1			2012	0.0					2.1
Aminoglycoside	Gentamicin	0	0	0				5.3	19.1	65	10.6						
Totrografino	Totroguelino	61.76%	60.0%	61.7%			5.3	14.9	15.9		2.1		-	11		6.4	E4 2
Tettacycline	i eti acyclille	(21)	(36)	(57)										1.1		0.4	54.5
Lincocamida	Clindomyoin	0	3.33%	2.13%		3.2	20.2	46.8	23.4	4.3		_			2.1		
Lincosamide Clindamycin	Chindaniyeni	0	(2)	(2)											2.1		
Ketolide Telithromyc	Talithromusin	0	3.33%	2.13%	2.1			2.2	4.3	39.4	21.0	17		2.1			
	Tentifolityciii	0	(2)	(2)	2.1			3.2			31.9			2.1			
Dhaniaal	Florfonicol		1.67%	1.06%		11			0.1	15.0	70.2	0 5	1 1			11	
Phenicol	Flortenicol	0	(1)	(1)		1.1			2.1	13.9	70.2	0.0	1.1			1.1	

Table 3.3. Frequency (%) of resistance observed over antimicrobials and the distribution of MICs

*CLSI: Clinical and Laboratory Standards Institute

†The unshaded areas indicate the dilution range of the CAMPY plates used to test isolates. Single vertical bars indicate the breakpoints for resistance. Epidemiologic cut-off values (ECOFFs) were used for the breakpoints.

	All cases (n=94)								Μ	ichiga	an case	s (n=5	53)	
Characteristic	Total No	FQ- resα	p^{δ}	TET- res ^β	pδ	FQTET -res ^y	pδ	Tota l No	FQ- resα	pδ	TET- res ^β	pδ	FQTET -res ^y	pδ
Age														
≤4	26	4		18		3		12	0		9		0	
5~49	39	8	.89	21	.4	6	.93	22	2	.79	9	.12	1	1.0
≥50	29	6		19		4		19	1		12		1	
<u>Sex</u>														
Male	52	10	00	29	10	6	50	30	2	1.0	16	10	1	1.0
Female	39	7	.00	27	.19	6	.59	22	1	1.0	14	.40	1	1.0
<u>Race</u>														
Caucasin	60	12	74	36	02	10	1.0	40	3	57	21	1.0	2	1.0
Non-caucasian	17	4	./4	10	.95	2		4	0	.57	2	1.0	0	1.0
<u>Residence</u>														
Wayne	30	6		19		4		12	0		9		0	
Washtenaw	17	5	FF	11	FO	5	21	12	2	.34	7	.08	2	.11
Oakland	10	1	.55	4	.50	1	.21	7	0		1		0	
Other	30	4		18		2		22	1		13		0	
<u>Travel</u>														
Foreign travel	9	7		7		6		-	-		-		-	
Domestic travel	17	2	<.0001	11	.49	1	<.0001	11	0	1.0	6	1.0	0	1.0
No travel	42	3		24		2		41	3	1.0	24	1.0	2	1.0
<u>Animal contact</u> †														
Domestic animal	38	5	.19	22	.49	3	.077	30	2	1.0	16	.72	1	1.0
Livestock	7	0	.33	7	.037	0	.58	5	0	1.0	5	.05	0	1.0
Birds/poultry	6	0	.58	5	.39	0	.58	6	0	1.0	5	.20	0	1.0

 Table 3.4. Univariate analyses of potential factors associated with antimicrobial resistant *C. jejuni* infections among

 all cases (n=94) and cases from Michigan only (n=53)

Table 3.4. (cont'd)

Food consumption*														
Ground meats	33	6	1.0	19	.77	4	1.0	27	2	1.0	14	.61	1	1.0
Home chicken	30	1	.015	15	.06	0	.003	27	0	.12	13	.25	0	.12
Frozen chicken	14	1	.67	4	.01	0	.18	13	0	1.0	4	.04 6	0	1.0
Restaurant chicken	19	2	.70	10	.3	2	1.0	15	0	.54	6	.15	0	1.0
Raw milk	4	1	.44	3	.63	0	1.0	2	0	1.0	2	.49	0	1.0
Water at home														
Well	12	1	40	10	055	1	1.0	9	0	1.0	7	15	0	1.0
Others	49	8	.40	25	.055	5	1.0	37	3	1.0	18	.15	2	1.0
<u>Season</u>														
Summer	42	7	12	23	20	5	045	25	2	1.0	13	1.0	1	12
Winter	13	5	.15	10	.20	5	.045	8	1	1.0	4	1.0	1	.45

• Several variables were categorized differently than Table 3.2. in order to investigate and maximize the potential

associations with different antimicrobial resistant profiles of *C. jejuni* infections.

^αThe cases with fluoroquinolone (ciprofloxacin) resistant *C. jejuni* infections.

 $^\beta$ The cases with tetracycline resistant C. jejuni infections.

γ The cases with fluoroquinolone-tetracycline resistant *C. jejuni* infections.

 $^{\delta}$ From χ^2 test or Fisher's exact test
Table 3.4. (cont'd)

† The counts for animal contact and food consumption were not mutually exclusive for each category, thus *p*-value for each

category was calculated.

Table 3.5. Univariate and multivariate analyses of factors associated with fluoroquinolone resistant *C. jejuni* infections among all cases (*n*=94)

Chanastanistis	U	nivariate analy	sis	M	Multivariate analysis				
Characteristic	OR	95% CIα	р	OR	95% CI	р			
Foreign travel	35.7	5.78-220.38	<0.0001	40.79	5.34-311.50	0.0003			
Season (Winter)	3.27	0.92-11.58	0.1204	8.86	1.04-75.16	0.0456			
Age_years	-	-	-	1.038	0.99-1.09	0.1002			
Sex (Female)	0.92	0.3152-2.68	0.88	-	-	-			
Domestic animal contact	0.368	0.1015-1.33	0.19	0.26	0.041-1.659	0.1542			
Home prepared chicken	0.086	0.0095-0.78	0.015	-	-	-			

• Age-years is a continuous variable; not proper for univariate analysis used (χ^2 or Fisher's exact test)

 Consumption of home prepared chicken was a significant protective factor by univariate analysis; however it was not used for multivariate analysis because of the small sample size left when the characteristic was included in the base model.
^α Wald Confidence interval

Table 3.6. Characteristics of cases with ciprofloxacin-resistant and ciprofloxacin-

susceptible C. jejuni infections

Variable	Total cases	Cases with ciprofloxacin- resistant <i>C.</i> <i>jejuni</i> infections	Cases with ciprofloxacin - susceptible <i>C.</i> <i>jejuni</i> infections	<i>p</i> - value*
Age, years	29.16 (n=94)	33.28 (n=18)	28.19 (n=76)	0.41
Foreign travel within 7 days	13.85% (n=65)	58.33% (n=12)	3.77% (n=53)	< 0.0001
Domestic travel within 7 days	26.47% (n=68)	16.67% (n=12)	28.57% (n=56)	0.4
Hospitalization	27.71% (n=83)	37.5% (n=16)	25.4% (n=67)	0.33
Mean days in hospital	3.43 (n=21)	4.5(n=6)	3.0 (n=15)	0.22†
Duration of illness	9.44 (n=41)	11.78 (n=9)	8.78 (n=32)	0.12†
<u>Clinical symptoms</u>				
Diarrhea	98.78% (n=82)	100% (n=16)	98.48% (n=66)	0.62
Bloody diarrhea	40% (n=80)	37.5% (n=16)	40.63% (n=64)	0.82
Nausea	42.50% (n=80)	43.75% (n=16)	42.19% (n=64)	0.91
Vomiting	27.50% (n=80)	12.5% (n=16)	31.25% (n=64)	0.21
Abdominal pain	66.67% (n=81)	62.5% (n=16)	67.69% (n=65)	0.69
Fatigue	42.50% (n=80)	50% (n=16)	40.63% (n=64)	0.5
Headache	26.25% (n=80)	25% (n=16)	26.56% (n=64)	0.9
Chills	30% (n=80)	31.25% (n=16)	29.69% (n=64)	0.9
Bodyache	27.50% (n=80)	18.75% (n=16)	29.69% (n=64)	0.38
Fever	54.67% (n=75)	57.14% (n=14)	54.1% (n=61)	0.84

- The % frequency reported from total cases and the number of cases that were available for each variable are specified.
- From χ^2 test or Fisher's exact test († Student's *t*-test).

Table 3.7. Mutations in *gyrA* from fluoroquinolone-resistant (MIC $\ge 4\mu g/mL$) and fluoroquinolone-susceptible *C. jejuni*

isolates (MIC<4 µg/mL)

Strain	ст	MIC					Nucleo	otide mu	tation rel	ative to A	ATCC335	60 <i>gyrA</i> s	equence	
Suam	51	(µg/mL)	T84C	C117T	T174C	T228C	T234C	C257T	C257G	A258G	С330Т	A324G	T351C	T354C
TW16431	3574	32						NS						
TW16469	44	32						NS						
TW16455	2310	16						NS						
TW16398	982	8						NS						
TW16409	122	8						NS						
TW16402	45	0.25					S							
TW16435	45	0.25					S							
TW16399	353	0.06									S			
TW16445	353	0.06									S			
TW16463	45	0.06					S							
TW16464	45	0.06					S							
TW16401	468	0.12	S	S									S	S
TW16689	986	16						NS						
TW16706	464	16						NS						
TW16607	6	16				S			NS	NS	S	S	S	
TW16617	464	16						NS						
TW16493	982	8						NS						
TW16494	982	8						NS						
TW16638	48	8						NS						
TW16655	2083	8						NS						
TW16657	5221	8			S			NS						
TW16695	50	8						NS						
TW16698	464	8						NS						
TW16735	7010	8			S			NS						
TW16727	3510	4						NS						
TW16637	7009	0.12		S										
TW16628	4185	0.12	S	S									S	S
TW16514	353	0.12									S			
TW16515	353	0.12									S			
TW16629	45	0.12					S							

Table 3.7. (cont'd)

TW16632	45	0.12		S		
TW16656	2875	0.12	S			
TW16725	45	0.12		S		
TW16648	353	0.06			S	
TW16709	353	0.06			S	
TW16719	21	0.06			S	

• S; synonymous mutation, NS; non-synonymous mutation

• The list is ordered by year, then MIC; high to low

Table 3.8. Mutations	s in the 23s rRNA	genes from Multi	ple Drug Resista	nt <i>C. jejuni</i> isolates
		0	F	

		MIC (ug/ml) for	Posistanco	Nucleotide mutation relative to <i>C. jejuni</i> ATCC33560 23s rRNA						
Strain	ST	Azithromycin	profile	A2074T	C2097T	C2113T	T2172G	T2252C		
TW16607	6	≥64	AziCipEryNalTel	NS		S	S			
TW16657	5221	≥64	AziCipEryNalTel	NS	S	S	S			
TW16735	7010	0.12	CipNalTet			S	S	S		

• S; synonymous mutation, NS; non-synonymous mutation

Figure 3.1. GIS map of Michigan by county showing the frequency of *Campylobacter* cases reported in 2011-2012



• The stars represent the location of four hospitals where the samples were collected.

Figure 3.2. Distribution of MICs for ciprofloxacin, azithromycin, and tetracycline





Figure 3.3. Phylogenetic tree of STs found in the study with the antimicrobial

resistance pattern, CC, and travel information



Figure 3.3. (cont'd)

- The numbers at the branch represent the sequence types (STs) found in this study.
- The numbers at the nodes represent the bootstrap value from 1,000 replications.

Figure 3.4. Number of isolates assigned to each Clonal Complex (CC) observed in the study; stratified by the travel history of the cases



Figure 3.5. Recombination among STs from all isolates (*n*=94) based on the 144 parsimonious informative sites (PHI=0.0)





Figure 3.6. Recombination among STs from Michigan showing 5 clusters

- star sign (*) represents the STs from cases with travel history in Michigan; the travel locations were far from the sample collection sites.
- R represents a ST which was composed of one isolate that were resistant to 4 different classes of antimicrobials (Fluoroquinolone, Macrolides, Ketolides,



Figure 3.7. Phylogenetic tree of STs from Michigan (*n*=53) showing 5 clusters

• A total of 13 STs were classified as singletons.

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CHAPTER4

Campylobacter jejuni isolates from cattle in Michigan: genetic diversity,

antimicrobial resistance profiles, and impact on public health

ABSTRACT

A total of 168 *Campylobacter* isolates were recovered from 220 fecal samples from three cattle farms in Michigan. The animal level prevalence, 76.4%, was significantly higher than previous studies, warranting the need to further investigate the prevalence and associated risk factors of *Campylobacter* among cattle in Michigan. Furthermore, high rates of resistance to tetracycline (83.7%) were observed, especially from Farm C, as 95% of the total isolates were resistant to tetracycline. The genotyping data revealed that the isolates from Farm C were mostly assigned to multilocus sequence type (ST)-459, which has been identified as a cattle-specific ST. Resistance to ciprofloxacin was also observed in 16.3% (*n*=22); all of the ciprofloxacin resistant isolates were also resistant to tetracycline. Isolates with resistance to ciprofloxacin, nalidixic acid, and tetracycline more commonly belonged to ST-1244, ST-7679, and ST-929. ST-1244 was mostly observed in Farm B, while ST-7679 was observed only in Farm A, indicating that these resistant clones are diverse and unique to specific environments. ST-982, a genotype associated with tetracycline resistance in the previous study with human *C. jejuni* isolates, were prevalent in cattle. The majority (86.4%) of cattle ST-982 isolates also had resistance to tetracycline, suggesting that cattle may be an important source of tetracycline resistant ST-982 *C. jejuni* infections in humans in Michigan. Seven additional STs were shared between humans and cattle, and all of the STs were more closely related to cattle-derived isolates in the phylogenetic analysis. Resistance to clinically important drugs such as ciprofloxacin, azithromycin, and erythromycin, were more common in cattle-specific STs like ST-1244, ST-7679. Yet, the phylogeny suggests diversification of resistant clones in cattle, which warrants continuous monitoring of *Campylobacter* resistance in this important reservoir.

INTRODUCTION

Campylobacter is one of the most common causes of human gastroenteritis in the U.S., estimated to affect about 1.3 million people annually.¹ The majority of *Campylobacter* isolated from patients with gastroenteritis is *C. jejuni* (89%) and *C. coli* (8%).² The infection is usually self-limiting, but in severe cases, antibiotic treatment with fluoroquinolones and macrolides is required. Additionally, *C. jejuni* infections can lead to serious, long-term sequelae like Guillain-Barré Syndrome³ and reactive arthritis,⁴ causing considerable morbidity and economic impact.⁵

Campylobacter is a zoonotic pathogen, which can asymptomatically colonize the intestines of various food animals, i.e., chickens, cattle and pigs.^{6–8} Previous studies have been more focused on chickens, as they are considered to be the major reservoir for human campylobacteriosis.^{9,10} However, recent studies utilizing molecular tools, i.e. multilocus sequence typing (MLST), and statistical modeling like asymmetric island model¹¹ and STRUCTURE,^{12,13} have shown that cattle are an important source for human infections. Indeed, the source attribution studies recently conducted in Finland and the U.K. both found that cattle contributed equally as chickens as the source for human campylobacteriosis.^{11,14} Additionally, a study conducted by CDC this year reported that dairy products, mostly raw milk and cheese, contributed to 66% of the campylobacteriosis outbreaks in the U.S.¹⁵

In the U.S., cattle have been shown to be a significant reservoir for *Campylobacter*, with the prevalence ranging from 81% to 100%, and 38% to 51% at the herd and individual animal level, respectively.¹⁶⁻¹⁹ Furthermore, *Campylobacter* shed by

cattle can contribute to contamination of not only their products, i.e. milk and meats, but also of the environment including run-off water from farming, processing operations and soil. These environments represent additional sources for human infections. One study, for example, has found identical biotypes of *C. jejuni* recovered from dairy cattle and ground water, which is suggestive of transmission to or from the environment.²⁰ Although not as frequent as food consumption, swimming and drinking contaminated water have also been identified as important routes of *Camplylobacter* transmission to humans.^{20,21}

Another major concern with regard to *Campylobacter* is the increasing trend of antimicrobial resistance, especially to drugs of clinical importance for humans, i.e. fluoroquinolones and macrolides. The association between the use of fluoroquinolones in poultry and the increasing resistance in human isolates has been investigated and resulted in a ban on use in poultry in the U.S.²² In spite of this effort, persistence and increasing frequencies of fluoroquinolone resistant *Campylobacter* have been reported in both chickens and humans.²³ Importantly, experimental studies have noted an increased fitness of fluoroquinolone resistant *C. jejuni* in chickens, the natural host, even without the selective pressure, or use of the antimicrobial.²⁴ Not much effort, however, has been put into investigating other potential reservoirs, i.e. cattle and pigs, which also serve as important sources for antimicrobial resistant *Campylobacter* infections in humans.

In cattle, fluoroquinolones and macrolides are often administered for treatment purposes as well as disease prevention and growth promotion. Fluoroquinolones such as enrofloxacin, have been licensed for use in beef cattle since 1998, and macrolides

including tulathromycin and erythromycin, are commonly used for treating respiratory diseases in both beef and dairy cattle. The use of tetracyclines, like chlortetracycline or oxytetracycline, is also common in cattle herds. According to the national study conducted by the U.S. Department of Agriculture (USDA) in 2007, over one-half of operations (57.5%) were feeding medicated milk replacer, often containing tetracycline, to calves and pre-weaned heifers.²⁵ Also, tetracycline was the drug most commonly used for treating lameness. Resistance frequencies to these agents in different cattle populations, however, have not been well studied.

Previously, we conducted a molecular epidemiological study of human *C. jejuni* isolates in Michigan, which investigated antimicrobial resistance frequencies, resistance mechanisms, and genetic diversity. One of the most notable findings was the significantly higher rate of tetracycline resistance compared to the report published by the National Antimicrobial Resistance Monitoring System (NARMS).²³ Furthermore, resistance to tetracycline was significantly higher in *C. jejuni* isolates from cases reporting contact with livestock, specifically cattle, prior to the onset of symptoms. A specific genotype, sequence type (ST)-982 as determined by MLST, was linked to both tetracycline resistance and livestock contact, implying that a tetracycline resistant, pathogenic clone may be circulating in the cattle population. To investigate this possibility, we sought to examine the genetic diversity and antimicrobial resistance profiles of 135 *C. jejuni* recovered from cattle in Michigan during the same time frame as the human isolates were previously characterized. We hypothesized that there was a high prevalence of *C. jejuni* among cattle in Michigan, and that the isolates had similar resistance levels to fluoroquinolones and tetracycline when compared to human

isolates. Furthermore, we hypothesized that similar genotypes with identical antimicrobial resistance profiles would be recovered from both cattle and humans in addition to a subset of cattle-specific genotypes, which are more readily transmitted within and across herds.

Materials and Methods

Sample and data collection

An epidemiologic study was conducted between May 2011 and October of 2012 to investigate fecal shedding of Shiga toxin producing *E. coli* in cattle,²⁶ and a subset of the samples and data were used in this study. Overall, 220 fecal grab samples from one dairy and two beef operations in mid-Michigan were collected between July and August 2012 for *Campylobacter* culture. A questionnaire, which was administered by personal interviews with each of the farm owners or managers, was used to obtain data regarding farm demographics, farm management practices, and herd health management strategies. This study was approved by the Institutional Animal Care and Use Committee of Michigan State University (AN12/10-223-00).

Isolation and identification of campylobacter jejuni

Ten µl of each fecal sample was directly plated on blood agar plates containing cefoperazone (20µg/ml), vancomycin (20µg/ml) and amphotericin B (4µg/ml) for 48 hours at 37°C in a microaerophilic condition using the Oxoid[™] CampyGen (Thermo scientific, Waltham, MA). Three single colonies were subcultured from each sample based on morphology and appearance while focusing on small pinpoint gray colonies without hemolysis. After incubation for 48 hours at 37°C in microaerophilic conditions, DNA was extracted from each single colony culture using the Wizard® genomic DNA purification kit (Promega, Madison, WI). The identification of *Campylobacter* and the species was performed using the extracted DNA by multiplex PCR as described previously.²⁷ The

confirmed isolates were stored in trypticase soy broth with 10% glycerol at -80°C and extracted DNA was stored at -20°C until further testing.

Phenotypic antimicrobial susceptibility profiling

The minimum inhibitory concentrations (MICs) of nine antimicrobials were determined by a standard broth microdilution test following the guidelines of Clinical and Laboratory Standards Institute (CLSI).²⁸ A 96-well plate (Sensititre, Trek Diagnostic Systems, Thermo Fisher Scientific Inc., Cleveland, OH) was used for each isolate following the manufacturer's instruction.²⁹ Tested antimicrobials included ciprofloxacin (fluoroquinolone), nalidixic acid, azithromycin (macrolide), erythromycin (macrolide), tetracycline, florfenicol, telithromycin, clindamycin, and gentamicin. *C. jejuni* ATCC 33560 was used as the quality control strain for every batch, and the breakpoints for each antibiotic were determined using the epidemiologic cut-off values (ECOFFs) following the guidelines of European Committee on Antimicrobial Susceptibility Testing³⁰ and NARMS.²³ Multiple drug resistance was defined as resistance to two or more classes of antimicrobials tested.

Multilocus Sequence Typing (MLST)

MLST was performed using the primers listed on the *C. jejuni* and *C. coli* PubMLST website (http://pubmlst.org/campylobacter). The amplification of seven genes was performed using the Kapa2G fast PCR kit (KapaBiosystmes, Boston, MA) with a cycling condition of: one cycle of denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15s, annealing at 60°C for 15s, extension at 72°C for 5s, and a final

extension step at 72°C for 2 min. The amplified products were cleaned using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced at the Research Technology Support Facility at Michigan State University. The sequences were assembled and checked for overall quality using the SeqMan program in the Lasergene software suite (DNASTAR Inc., Madison, WI). Alleles, STs, and clonal complex (CC) assignments were made using the PubMLST (www.publmlst.org/campylobacter/) database for each isolate. New alleles (*n*=2) and STs (*n*=8) found in this study were submitted to the database.

Repetitive Sequence-based (Rep)-PCR

Rep-PCR fingerprinting was conducted using a set of primers as previously described³¹ (Table 4.1.) to enhance the ability to assess the genetic diversity of *C. jejuni* from cattle within the same herd. All template DNA concentrations were standardized to 25ng/µL prior to PCR and 0.8µM of each primer was used. KAPA2G Fast Readymix was used with the following cycling conditions: one initial cycle at 95°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 52°C for 1 min, and extension at 65°C for 10 min, with a single final extension cycle at 65°C for 20 min. The amplified products were separated by electrophoresis at 80 V for 2 hours using a 1.5% agarose gel. The fingerprint patterns were analyzed visually and with Bionumerics ver. 5.10 (Applied Maths, Inc., Austin, TX). Banding patterns were examined using the Dice coefficient with a 2.0% band position tolerance for calculating the similarity matrices. Dendrograms were created using the unweighted pair group method with arithmetic averages (UPGMA).

Determination of presence and location of *tet(0)* gene

The presence of *tet(O)*, the gene responsible for conferring tetracycline resistance in *Campylobacter*, was identified by a PCR reaction as previously described.³² The isolates that harboured *tet(O)* were further tested for the location, i.e. inserted in chromosome or plasmid (pTet), using previously described primers and condition.³³

Data analyses

The map of cattle numbers in Michigan in 2012 was generated by ArcMap GIS software (version 10.2; ESRI, Redlands, California) using the data from USDA Census of agriculture.³⁴

All the statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). Differences in the frequencies of antimicrobial resistance across ST, CC, and other variables including different farms were examined using χ^2 and Fisher's exact tests for small sample sizes; a *P*<0.05 was considered significant.

A Neighbor joining tree (*p*-distance) with 1,000 bootstrap replications was constructed in MEGA 6³⁵ to identify the evolutionary relationships between isolates. Clusters were characterized by STs that grouped together with >75% bootstrap support, and were further evaluated for the genetic recombination using Splitstree4.³⁶

RESULTS

Description of farms

The three farms sampled for *C. jejuni* were located in three different counties in mid-Michigan (Figure 4.1.). The geographical information system (GIS) map shows the cattle numbers reported by the U.S. Department of Agriculture at the county level for year 2012 in Michigan.³⁷ According to the questionnaire data obtained at the time of sampling, Farm A had approximately 5,000 animals in a 2 mile radius, while Farm B and Farm C had 500 and 100 animals in the vicinity, respectively. Farm A, a dairy operation, housed approximately 530 animals, and Farm B and Farm C, beef operations, had 83 and 75 animals, retrospectively. Farms A and C had cattle that were crossbred, while Farm B consisted of Holsteins. All three farms reported using antiparasitic drugs, i.e. Cydectam (moxidectin), Dectomax (doramectin) as a preventive measure, but only Farm C reported the use of chlortetracycline in the feed or water for new cattle upon their arrival and after. Other antimicrobials including ceftiofur (Excede, Excenel, Exceed), florfenicol (Nuflor) and macrolides (gamithromycin (Zactran), tulathromycin (Draxxin)), were used to treat respiratory disease on all three farms. In addition, Farm B reportedly used oxytetracycline (Oxytet200) for treating both foot infections and arthritis, while Farm C used a macrolide (Draxxin) for the same treatments. Farm A used oxytetracycline and ampicillin (Polyflex) together for treating cases of clinical mastitis and metritis. All three farms reported having various animal species in the farm environment including starlings, pigeons, raccoons, rodents, etc. For cleaning, Farm C sprayed disinfectant approximately once per 6 months,

whereas Farms A and B did power washing once a week or as needed, respectively. The information collected through questionnaire is summarized in Table 4.2.

Prevalence of Campylobacter in three cattle herds in mid-Michigan

The overall prevalence of *Campylobacter* isolated from three farms was 76.4% (168/220). Only one *C. coli* isolate was recovered from Farm A, while the remaining 167 isolates were characterized as *C. jejuni*. Sample collection from Farm A was conducted in July while the farms B and C were sampled in August. Almost all of the animals in Farms B and C were sampled, while only 12% of the total animals were sampled at Farm A. The prevalence of *C. jejuni* was the highest at Farm A (85.7%), followed by Farm C (84.0%) and Farm B (61.0%) (Table 4.3.). Differences in prevalence between Farms A and B, and between Farms B and C were statistically significant (*p*<0.05); a significantly lower prevalence was observed for Farm B compared to the other two sites.

Antimicrobial resistance profiles of C. jejuni isolates

After initial culture and speciation, 135 of the 167 (80.8%) *C. jejuni* isolates were viable and could be tested for susceptibility to nine antimicrobials. Specifically, 25, 50, and 60 isolates from Farms A, B, and C, respectively, were tested. Overall, 22 isolates (16.3%) were susceptible to all nine antimicrobial agents tested (pan-susceptible), while 113 isolates (83.7%) showed resistance to one or more agents. The highest frequency of resistance was observed for tetracycline (83.7%), followed by nalidixic acid and ciprofloxacin. Resistance to macrolides, azithromycin and erythromycin, was observed in two isolates (1.5%), which were also resistant to other classes of antimicrobial agents and

were classified as multiple-drug resistant (MDR). The majority of MDR isolates (n=21; 91.3%) were resistant to ciprofloxacin, nalidixic acid, and tetracycline (CipNalTet). One additional isolate was resistant to azithromycin, erythromycin, nalidixic acid, and tetracycline and another isolate was resistant to azithromycin, erythromycin, ciprofloxacin, nalidixic acid, telithromycin, and clindamycin. Differences in resistance profiles were also observed between farms (Figure 4.2.). In detail, while more than half (n=15; 60%) of isolates recovered from Farm A were pan-susceptible, Farm B had only five (10%) pan-susceptible isolates plus a high rate of CipNalTet resistance (n=15; 30%). On the other hand, most of the isolates (n=58; 96.7%) recovered from Farm C were resistant to at least one agent, and all were resistant to tetracycline with the exception of one MDR isolate. The overall frequency of antimicrobial resistant *C. jejuni* differed significantly between farms: the dairy farm, Farm A, had a significantly lower proportion of antimicrobial resistant *C. jejuni* compared to the two beef farms (Table 4.3.).

To define the mechanism of tetracycline resistance in the *C. jejuni* isolates recovered, PCR was used to determine the presence and location of *tet(O*). All 113 tetracycline resistant isolates harbored *tet(O)* and 28 (25.2%) of these were inserted in pTet plasmids.

Genetic diversity and frequency of C. jejuni genotypes in Michigan cattle

MLST was used to investigate the diversity of *C.jejuni* isolates recovered from cattle in all three farms and to make comparisons to the human-derived isolates. A total of 22 different sequence types (STs), including eight novel STs, were represented among the 135 *C. jejuni* isolates recovered from cattle in Michigan. Eighteen of the 22 STs were assigned to six previously defined clonal complexes (CCs), while the remaining four STs were classified as singletons. The Neighbor joining algorithm grouped all 22 STs into five clusters with significant bootstrap support (>75%) (Figure 4.3.). The four STs, which were classified as singletons via PubMLST (CCs), grouped together into two distinct clusters that also contained STs representing other previously defined CCs. Multiple CCs were also found to group together within a given cluster. For example, Clusters IV and V contained CC-42 and CC-403, and CC-257 and CC-61, respectively, while all of the STs comprising Cluster I were assigned to CC-21. Because more than one CC often clustered together with >95% bootstrap support, we classified all isolates in this study as belonging to Clusters I-V. In this classification, only two isolates were classified singletons and did not group with isolates in one of the five clusters. Although the Neighbor-net analysis, both on all sites and 162 parsimonious informative sites, indicated significant evidence of recombination among 22 STs (pairwise homoplasy index (PHI)=0.0), the five clusters identified in the Neighbor-joining phylogeny were still evident (Figure 4.4.).

Among the 135 *C. jejuni* isolates, the most prevalent STs were ST-459, ST-982, which were widespread in all three farms (Figure 4.3.). ST-1244, ST-806, ST-922, and ST-933 were found in both Farms A and B, while ST-929 was shared between Farms B and C. The remaining 15 STs identified were exclusive to specific farms and most were represented by only one isolate. Notably, ST-5538 (n=6) was found only at Farm A, and two of the novel STs, ST-7679 (n=3) and ST-7696 (n=2), were recovered only from Farms A and C, respectively. Among the previously defined CCs, CC-42 was the most prevalent clonal complex (n=58; 42.9%) found in this study, followed by CC-21 (n=33; 24.4%) and CC-61(n=17; 12.6%). These predominant CCs belonged to three distinct clusters, namely Clusters IV, I, and V, respectively.
Association between phylogenetic lineage and antimicrobial resistance profiles

The most prevalent ST found in the study, ST-459 (n=57), was significantly associated with tetracycline resistance (p<0.0001; OR=22.0; 95% CI=2.9-168.9) as 55 ST-459 isolates (96.5%) were resistant to tetracycline only (Figure 4.5.). Most of the 22 isolates assigned to ST-982 (n=19; 86.4%) were also resistant to tetracycline only, but because of the high proportion of tetracycline resistance among ST-459 isolates, the association was not significant in the overall analysis (Fisher's p=0.12). Twenty three (41.8%) of the 55 tetracycline resistant ST-459 isolates had *tet*(*O*) inserted in the pTet plasmid compared to only two of the 19 (10.5%) tetracycline resistant ST-982 isolates.

For fluoroquinolone resistance, isolates belonging to ST-1244 were more likely to be resistant to ciprofloxacin and nalidixic acid (p<0.0001, OR=97.1, 95% CI=18.72-503.80). In detail, 14 of the 16 ST-1244 isolates were resistant to both ciprofloxacin and nalidixic acid. Thirteen of these isolates were also resistant to tetracycline, yielding a significant association between ST-1244 and CipNalTet resistance (p<0.0001; OR=60.1, 95% CI=14.16-255.28). Similarly, isolates assigned to ST-929 had a higher odds of resistance to CipNalTet together (p<0.05; OR=9.33, 95% CI=1.46-59.78) as did isolates assigned to ST-7679 (n=3; Fisher's p<0.01). Two genotypes, ST-5538 and ST-922, were significantly associated with susceptibility to all antimicrobials examined.

Because of the associations identified with specific STs and resistance profiles, similar associations were identified across the clusters identified in the Neighbor joining phylogeny (Figure 4.5.). Cluster IV, for example, mostly consisted of ST-459 (n=57) isolates, was significantly associated with tetracycline resistance (p<0.0001; OR=13.1; 95% CI=2.9-58.5). Similarly, Cluster V was associated with CipNal (p<0.0001; OR=50.5; 95% CI=14.4-

177.5) and CipNalTet resistance (p<0.0001; OR=41.6; 95% CI=12.1-143.1) as ST-1244 (n=16) comprised the majority (66.7%) of isolates within this cluster. On the other hand, Cluster II, mostly comprising ST-5538 and ST-922, showed a significant association with pan-susceptibility (Fisher's, p<0.0001). A similar association was observed for Cluster III with pan-susceptible profile (Fisher's, p<0.005), though the number of isolates in both Clusters II and III was small.

DNA fingerprinting analysis of *C. jejuni* isolates to investigate genetic diversity and transmission

Repetitive-PCR (rep-PCR) was performed on all 135 *C. jejuni* isolates to assess the genetic diversity of isolates that were assigned to the same STs, and examine transmission of *C. jejuni* within and between farms. The fingerprinting technique, which amplifies the specific repetitive sequences interspersed throughout the *Campylobacter* genome, was correlated with MLST results (Figure 4.6.). Specifically, the isolates assigned to ST-459 and ST-982 formed two major clusters by the unweighted pair group method with arithmetic average (UPGMA), while the pan-susceptible genotypes (ST-922 and ST-5538) grouped into distinct clusters.

When each of the clusters identified in the UPGMA analysis were examined separately, ST-459 had a common banding pattern (459-F) consisting of nine bands on average regardless of resistance profile and farm. Several unique patterns were also observed, i.e., US932, US984, US1040, US1060, US1078, US1096, US1112, which differed by 1-8 bands and were designated as patterns 459-A to 459-E (Figure 4.7.). ST-42 had the same banding pattern as the predominant ST-459 pattern (459-F). In addition, the ST-1244

isolates that clustered together with ST-982 on the overall analysis, had an additional band upon manual examination (Figure 4.8.; red arrow). Importantly, all three CipNalTet resistant isolates belonging to ST-7679 were included in the ST-982 cluster and were indistinguishable from the predominant ST-982 banding pattern (982-G). In fact, ST-7679 and ST-982 differed by only one single nucleotide polymorphism (SNP) in one of the 7 housekeeping loci by MLST. Two ST-982 isolates from Farm A that had the same banding pattern (982-G) were also resistant to CipNalTet, strongly suggesting a diversification of a CipNalTet resistant ST-982 in Farm A. Among the 22 ST-982 isolates, seven different banding patterns were observed, while only two patterns were identified for the 16 ST-1244 isolates: The predominant patterns for three STs, 459-F, 982-G, 1244-A, were observed at each of the three farms, whereas the unique patterns were confined to each farm. No association was observed between the banding patterns and resistance profiles.

Genetic relatedness of *C. jejuni* isolates from humans and cattle, and the association with antimicrobial resistance

To determine the genetic relatedness between *C. jejuni* isolates from humans and cattle, we also constructed a Neighbor joining phylogeny with all STs recovered from this study (*n*=22) as well as the previous study on human *C. jejuni* isolates (*n*=54). A total of eight STs, ST-8, ST-21, ST-982, ST-806, ST-922, ST-459, ST-42, and ST-929, were observed in both humans and cattle (Figure 4.9.). Although the bootstrap support was not high enough to identify specific clusters, the more closely related STs were assigned to same CCs by the PubMLST *C. jejuni* database. Notably, four of the eight overlapping STs, i.e. ST-21, ST-8, ST-982, ST-806, were assigned to CC-21. Among the new STs recovered from cattle, ST-

7679 was closely related to ST-982, while ST-922 was related to ST-7694 and STs 7693 and 7763 were related to ST-806. Similarly, ST-929 was included in a small cluster comprised of three STs from cattle (ST-1244, ST-3351, and ST-7696).

To better elucidate the evolutionary relationships of *C. jejuni* isolates recovered exclusively from Michigan, we constructed an additional phylogenetic tree comparing only the STs (n=35) that were isolated from Michigan patients to those recovered from cattle (n=22). STs from patients reporting any travel outside Michigan were excluded from this analysis. In the Neighbor joining phylogeny, the bootstrap values were high enough to support several clusters, including the clusters previously identified in both human and cattle isolates separately (Figure 4.10.). The same eight STs, as described above, were shared between human- and cattle-derived isolates in the analysis. Notably, three of these eight shared STs were included in the clusters that were observed both among human and cattle isolates, and three STs were included in cattle-specific clusters.

We also compared the antimicrobial resistance profiles of the eight STs shared between humans and cattle. Notably, similar resistance profiles were observed among isolates from each species (Figure 4.11.). Isolates belonging to ST-21 were pan-susceptible, while ST-42 and ST-459 isolates were predominantly tetracycline resistant. ST-982, which consisted of ten isolates from humans and 22 from cattle, shared a similar resistance pattern as well. In detail, the majority of isolates (81.3%) representing ST-982 were resistant to tetracycline only, however, two ST-982 isolates from both species were CipNalTet resistant. Unlike the human study, ST-982 isolates recovered from cattle did not show a significant association with tetracycline resistance. However, when the ST-459 isolates were excluded from the analysis, the ST-982 isolates from cattle were significantly

associated with tetracycline resistance compared to all other STs from cattle (n=20). Combining all of the susceptibility and genotyping data from cattle (n=135) and humans (n=94) confirmed the associations identified separately in both species. Specifically, ST-982 was significantly associated with tetracycline resistance (p<0.01; OR=6.11), while STs 21 and 922 were more likely to be pan-susceptible (p<0.01). ST-464 and ST-1244, which were STs found exclusively in humans and cattle, respectively, were significantly associated with CipNal (p<0.0001) and CipNalTet (p<0.0001) resistance. The same was true for ST-459 and resistance to tetracycline (p<0.0001; OR=29.26).

DISCUSSION

It has been well established that *Campylobacter* are prevalent in cattle in the U.S., with the herd level prevalence up to 100% reported. ¹⁶⁻¹⁹ The prevalence at the animal level has been reported to range between 38% and 51%, however, we found 76% (168/220) of the samples to be positive for *Campylobacter*, surpassing the range significantly (*p*<0.05). Nonetheless, the optimal culture-based method for *Campylobacter* was not unified across studies, and in fact, different media and methods have been used, thereby limiting a comparison of prevalence estimates across studies. The common step included in the previous literature, that is different from our method, was use of a broth, e.g. Preston broth,³⁸ Campy-thio broth,^{16, 18} or a solution, i.e. phosphate-buffered saline,^{19, 39} buffered peptone water,¹⁷ for enrichment or dilution before streaking on agar plates. We directly plated fecal samples on a selective agar that was prepared in-house using 5% sheep blood and three antibiotics: cefoperazone, amphotericin B, and vancomycin. Also, we processed our samples on the same day of each collection, within a few hours. *Campylobacter* is known for its susceptibility to low temperatures and unfavorable atmosphere conditions.^{40, 41} We therefore hypothesize that the same day processing strategy and direct plating method could have contributed to the higher rate compared to prior studies. Lastly, all three farms were located in central Michigan (Figure 4.1.), where no study had been done to investigate *Campylobacter* prevalence in cattle before.

A very distinct predominance of certain genotypes, CC-42, CC-61, and CC-21 were observed among cattle isolates. Similar findings have been reported from other European countries and in the U.S, suggesting the high adaptation of these genotypes to cattle.^{14,42,43}According to the PubMLST database, previously in the U.S., cattle, cow milk and

the farm environment were the main sources of CC-42 and CC-61, comprising 84.7% (61/72), 79.2% (42/53) of the total reported, respectively. CC-21 has been reported from more diverse sources, including sheep and chicken, but the major sources were cattle, cow milk (46.1%) and human clinical cases (30.56%). There were also several human cases reported with CC-42 and CC-61 isolates in the database, implicating the high possibility of these cattle-adapted genotypes being transmitted to humans. Although the previous studies support the significant link between these genotypes and cattle, it is also possible that the genotypes are transmitted between farms. The questionnaire shows that all three farms had contact with other animals, including dogs, cats, and wild animals like starlings, pigeons, raccoons and deer etc. A recent study conducted in Ohio found the same genotypes shared between cattle and starlings in the area,⁴³ suggesting the possibility of starlings being involved in transmission of the pathogen between cattle operations.

Antimicrobial susceptibility profiling showed that 84% (113/135) of *C. jejuni* isolates had resistance to one or more antimicrobials tested. The highest resistance rates were observed for tetracycline, which was observed in all of the resistant isolates (*n*=113). A significant difference, however, was observed in the frequency of tetracycline resistant *C. jejuni* observed between farms. Farm A had the lowest frequency (16%), while 95% of the isolates recovered from Farm C were resistant to tetracycline. Importantly, Farm C, a beef operation, was the only farm reporting use of chlortetracycline as a preventive measure. Specifically, the antimicrobial was added to the water upon arrival of a new group of animals, and was added continuously at 2 grams/head/day for 5 days every month. Farm B, another beef operation which had a 58% frequency of tetracycline resistance, was using oxytetracycline for treatment of foot infections and arthritis. These data are consistent with

prior studies that have documented high levels of tetracycline resistance in *C. jejuni* from cattle given therapeutic and subtherapeutic doses of the antimicrobial agent.^{44,45} Due to the low sample size (n=3) in this study, however, we could not perform a statistical analysis to confirm the link between the use of tetracycline and the frequency of resistance at the farm level. Nonetheless, the high rate of tetracycline resistance observed (84%) and the frequent use of tetracycline at these farms warrants further studies to investigate the association between farm management practices in cattle operations and the frequency of antimicrobial resistant *Campylobacter*. The frequency of macrolide (2%) resistance observed in this study was similar to prior cattle studies, which have ranged from 0 to2.9%.^{16,46} However, a higher frequency of fluoroquinolone resistance, 16% compared to 0.6-5.0%, was observed. We sampled virtually all cattle residing on Farms B and C, thus the observed frequency represents the point prevalence at each location. Farm B had the highest frequency of fluoroquinolone resistant isolates (69.6%; 16/23), while only one resistant isolate was recovered from Farm C. Based on the questionnaire data, none of the farms reported current use of fluoroquinolones, suggesting that resistance is maintained in the population in the absence of antimicrobial use, an important selective pressure. Indeed, resistance to fluoroquinolones typically involves a single point mutation in the *gyrA*, and it has been documented both epidemiologically and experimentally, that there is an increased in vivo fitness in fluoroquinolone resistant C. jejuni isolates even when the selective pressure is removed,²⁴ allowing the resistant isolates to persist and flourish.

Incorporating the genotyping data with the susceptibility data, we observed several interesting associations between specific STs and resistance. These associations further contributed to the associations observed in CC and Cluster level. Previously, a significant

link between the PFGE pattern and antimicrobial resistance profile have been reported for *C. coli* isolates from cattle in Washington,⁴⁷ and a study with human *C. jejuni* isolates in Korea revealed some novel STs found in the study were associated with multiple drug resistance.⁴⁸ However, to our knowledge, this is the first time such evident association is observed among cattle *C. jejuni*, supported by statistical analysis.

We conducted rep-PCR to enhance the ability to assess the genetic diversity and further investigate the transmission of the same genotype circulating within and across farms. Rep-PCR is a fingerprinting technique, which has been evaluated as a highly discriminatory method for studying *Campylobacter*, examining interspersed repetitive sequences throughout the genomes of the isolates. ^{31,49} As shown in Figure 4.6., the banding patterns and ST showed a good correlation, suggesting that rep-PCR can be a good genotyping tool, especially in limited resource settings as it does not require a sequencing step. Furthermore, the banding patterns generated by rep-PCR showed higher discriminatory power than MLST, as we could observe several different banding patterns on the isolates with same STs. However, ST-7679 and ST-982 had indistinguishable patterns by rep-PCR, which had one SNP in one of the seven MLST loci. Notably, two ST-982 isolates that had the same banding pattern as ST-7679 also shared the same resistance profile of being CipNalTet resistant (Figure 4.8.). All of these isolates were also from Farm A, strongly suggesting a diversification of CipNalTet resistant ST-982 in Farm A. Although these isolates were confined in Farm A, as ST-982 was a widespread lineage found in both humans and cattle in our studies, this finding strongly warrants a further investigation and a constant monitoring of this antimicrobial resistant clone. Also, the predominant STs with predominant banding patterns, i.e. 459-F, 982-G, 1244-A, that had identical resistance

profiles were shared between farms, implicating that there are antimicrobial resistant *C. jejuni* clones circulating in cattle population across farms.

One of the main objectives in this study was to determine if *C. jejuni* isolates from cattle were the same genotypes with identical resistance profiles that we previously observed in the human isolates. The study with human *C. jejuni* isolates in Michigan showed a significantly higher rate of tetracycline resistance compared to the national report, and the resistance was significantly linked with a genotype, ST-982, and having a history of livestock contact, i.e. cattle. Among cattle isolates, 22 (16.3%) were assigned to ST-982, showing a high prevalence of the genotype among cattle population. Nineteen of the ST-982 isolates were resistant to tetracycline, however, an even bigger proportion of tetracycline resistance was attributed by ST-459 in the cattle study, but the overall analysis did not show the association as significant. When we combined all the data from both humans (n=94) and cattle (n=135), the association became significant (p<0.01; OR=5.96). Furthermore, only two of the tetracycline resistant ST-982 isolates from cattle had *tet(0)*, the gene that confers tetracycline resistance in *Campylobacter*, inserted in the plasmid, pTet, that is known to carry and transfer the gene between *Campylobacter*. This strongly suggests that there is a tetracycline resistant ST-982 clone, circulating in cattle, making a crossover to humans. There were seven other overlapping STs between cattle and humans, i.e. ST-21, ST-8, ST-806, ST-922, ST-459, ST-42, and ST-929. And upon the phylogenetic analysis with human isolates only from Michigan (n=35), we could see most of the shared STs were included in the clusters that were observed among cattle (Figure 4.10.). ST-459 and ST-42 were not included in a cluster, but based on the PubMLST database, most of the isolates were reported from cattle, cow milk, and the farm environment, suggesting a close

relation to cattle. There was one cluster, all of which were assigned to CC-21, that was observed both in humans and cattle separately. This cluster included three STs out of eight: ST-8, ST-21, and ST-982, implicating the importance to monitor the occurrence and antimicrobial resistance of CC-21, both in humans and cattle.

This is a cross-sectional study with sampling based on convenience. Thus, we acknowledge the prevalence of *Campylobacter* we observed in this study may not be the true prevalence in Michigan. Additionally, there is a significant limitation on making any temporal causal link between the description of the farms and the prevalence and antimicrobial resistance observed. However, the observation of exceptionally high prevalence of *Campylobacter*, specifically *C. jejuni*, and the observation on the use of tetracycline and the increased resistance warrants further studies to investigate the risk factors and the impact of using antimicrobials on the prevalence and antimicrobial resistance of *C. jejuni* among cattle in Michigan. We also acknowledge the small sample size that were included in some of the analyses to find significant associations between certain genotypes and the antimicrobial resistance profiles, i.e. ST-7679 and CipNalTet, ST-5538, ST-922 and pan-susceptible profile, warranting further investigations. However, the most important associations found in the study, ST-982 and tetracycline resistance, ST-459 and tetracycline resistance, ST-1244 and CipNalTet, involved good numbers of isolates, strongly suggesting the presence of resistant clones circulating in the cattle population. Furthermore, observation of eight STs, including ST-982, shared between humans and cattle, and the closer genetic relationships to cattle clusters suggest a high possibility of humans in Michigan acquiring *C. jejuni* infections from cattle. Along with a further study to elucidate the real genetic relatedness and evolutionary relationships of the isolates with

shared STs, preventive measures should be discussed to control the zoonotic transmission between the two species.

APPENDIX

Gene	Primer	Sequence	Size (bp)
Rep-PCR ³¹	ERIC1R	5'-ATGTAAGCTCCTGGGGATTCAC-3'	
	ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'	
tet(0) ⁴⁹	F-campy tetO	5'-GCGTTTTGTTTATGTGCG-3'	
	R-campy tetO	5'-ATGGACAACCCGACAGAAG-3"	579
tet(0) ⁵⁰	tetOF1	5'-TAG CCG TAT AGA TAA GGT TCG-3'	
(plasmid)	cpp6-R1	5'-CTG TGC ATA AAA TCA TAG AAT-3'	~3,500

Table 4.1. Primers used in the study for Rep-PCR and *tet(O)* amplification

		Farm#1	Farm#2	Farm#3
Demographic	Operation type	Dairy	Beef	Beef
	Number of animals	530	83	75
	Breed	Crossbred	Holstein	Crossbred
Preventive measuresAntibiotic use in feed or water		No	No	Yes (Chlortetracycline)
	Any direct fed microbials	No	No	Yes (Yeast mineral package
	Antiparasitic	Yes (Cydectam)	Yes (Dectomax)	Yes (Dectomax)
	Rumensin in the feed	No	Yes	Yes
Treatment ^a Respiratory Disea		Excede, Excenel, Nuflor (Calf)	Excede, Nuflor, Zactran, Draxxin	Draxxin
	Foot infection	Copper Sulfate	Oxytet 200	Draxxin
	Arthritis	Unknown	Oxytet 200	Draxxin
	Clinical mastitis/ metritis	Oxytetetracycline, Polyflex	N/A	N/A
Contact with	Fly control	Yes (Premise spray)	No	No
other species	Dogs	Yes	No	Yes
	Cats	No	Yes	Yes
	Birds	Yes (Starlings,	Yes	Yes
		Pigeons)	(Sparrows, Starlings)	(Sparrows, Starlings, pigeons)
	Other animals	Yes (raccoons, rodents, deer)	Yes (raccoons, rodents, skunks)	Yes (raccoons, rodents, skunks, opossum, weasel)

Table 4.2. Farm information obtained through the questionnaire

Table 4.2. (cont'd)

Cleaning	Method		Scrape;Wash/Power Wash, Spread lime	Wash/Power Wash	Spray a disinfectant
	Frequency	Feedbunks	Once a week	When needed	Once per 6 months
		Waterers	Once a week	20 per month	Once per 6 months
Environment	Temperature ^b		85 °F (73 – 97 °F)	68 ^o F (62 - 73 ^o F)	75 °F (65 – 84 °F)

^a Common remedies used.

^b Average temperature on the day of sampling with the minimum and maximum temperature observed on the date.

Farm	Collection date	Total number of animals	Total samples collected	Prevalence of <i>C. jejuni</i>	pa	Prevalence of antimicrobial resistant <i>C. jejuni</i>	p ^a
Α	7/23/2012	530	63 (11.89%)	85.7% (<i>n</i> =54)		40% (<i>n</i> =10/25)	
В	8/13/2012	83	82 (98.8%)	61.0% (<i>n</i> =50)	< 0.05†	90% (<i>n</i> =45/50)	<0.00001‡
С	8/27/2012	75	75 (100%)	84.0% (<i>n</i> =63)		96.8% (<i>n</i> =58/60)	

Table 4.3. Prevalence of *C. jejuni* and the frequency of antimicrobial resistance by farm

^a Hypothesis test for comparing proportion was used.

[†] The *p*-value represent the difference in Farm B relative to Farm A and Farm C.

‡ The *p*-value represents the difference of Farm A to Farm B and Farm C.

Figure 4.1. GIS map of cattle number in each county and the location of sampling sites



• The letters in circle represent the location of farms where the cattle *C. jejuni* isolates were collected.

Figure 4.2. Frequency of antimicrobial resistance profiles in *C. jejuni* isolates



recovered from three cattle herds

• The numbers within each bar indicate the number of isolates for each resistance profile.



Figure 4.3. Neighbor joining phylogeny of 135 *C. jejuni* isolates recovered from cattle

- The number of isolates (% of total) assigned to each ST, and the farms from which the STs were recovered are listed.
- * The eight novel STs identified in this population is marked with asterisk.



Figure 4.4. Recombination among STs from all *C. jejuni* isolates from cattle (*n*=135)



Figure 4.5. Antimicrobial resistance observed in the study stratified by ST and cluster

The resistance profile for other MDR is <azithromycin, erythromycin, tetracycline, nalidixic acid> for ST-459 (Cluster IV), and <azithromycin, erythromycin, ciprofloxacin, nalidixic acid, tetracycline, telithromycin, clindamycin> for ST-1244 (Cluster V).

Figure 4.6. Cluster analysis of Rep-PCR pattern of 135 *C. jejuni* cattle isolates using

ERIC primers

0

Isolate ST Farm AR profile* 8 8 US992 933 в Tet US1000 7692 Tet в US900 933 А в US1039 929 Cip, Nal, Tet US1040 459 в Tet US1003 933 в Tet US1096 459 С Tet US970 5538 A -1111 US972 A A 58 US893 42 Tet US982 в 459 Tet US1008 459 в Tet US1016 459 в Tet US1017 459 в US1027 459 в Tet US1034 459 в Tet US1036 459 в Tet US1038 459 в Tet US1051 459 в Tet US1055 459 в Tet US1062 459 c c Tet US1064 459 Tet US1066 459 c c Tet 1 1 US1072 459 Azi, Ery, Nal, Tet c US1074 459 Tet 459 с US1076 Tet 1 US1079 459 c Tet 1 1 US1080 459 С Tet US1083 459 С Tet US1087 459 c c Tet US1093 459 Tet c c US1100 459 Tet US1101 459 Tet US1103 459 с Tet US1114 459 C Tet US1115 459 С Tet ŝ c c 111 . US1116 459 Tet 459 1 US1119 Tet 1 c US1120 459 Tet US1128 459 с Tet 1 US1129 459 С Tet US1133 459 С Tet 1 US1134 459 С Tet US1081 459 С Tet c c US1095 459 Tet US1113 459 Tet 459 A US940 Tet в US1042 459 Tet US1057 459 в Tet 目的 US1058 459 в Tet US1098 459 С Tet c c US1123 459 Tet US1061 459 Tet с 459 US1094 Tet в US1001 806 Tet US984 459 в Cip, Nal, Tet US1044 459 в Tet US932 459 A Tet Ч US1125 929 с Tet в US1075 7696 Tet L US1060 459 Tet US1086 929 c Tet US1078 459 с Tet US1112 459 С Tet A C US952 922 US1070 459 Tet US1043 459 в Tet US1077 c 1.11 7696 Tet US1049 в 459 Tet E US1090 459 С Tet US1028 459 в Tet

Dice (Opt:2.00%) (Tol 2.0%-2.0%) (H>0.0% S>0.0%) [0.0%-100.0%] ERIC-USDA

Figure 4.6. (cont'd)



- AR: Antimicrobial resistance
- Tetracycline resistance mediated by plasmid is in red.



Figure 4.7. ST-459 cluster by rep-PCR

• Type was assigned manually based on the banding patterns.



Figure 4.8. ST-982 cluster by rep-PCR

• Type was assigned manually based on the banding patterns.

Figure 4.8. (cont'd)

• The red arrow indicates the additional band location observed for ST-1244 isolates, compared to ST-982 isolates.

Figure 4.9. Phylogenetic tree of STs found in humans and cattle



0.005

Figure 4.10. Phylogenetic tree of STs found in humans from Michigan only and cattle





Figure 4.11. Histogram of the antimicrobial resistance profile of STs found both in

humans and cattle

• NA represents CC not assigned.

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CONCLUSIONS AND FUTURE DIRECTIONS

Campylobacter was the most commonly reported food-borne pathogen in Michigan in the last decade (2004-2013). Descriptive epidemiology study conducted using the data from Michigan Disease Surveillance System showed an increasing trend of both *Campylobacter* incidence and hospitalization rate due to *Campylobacter*. Notably, different epidemiology of *Campylobacter* was observed for different age groups. In detail, highest incidence was observed for young children, < 5 years, in all years. The cases in this age group were more likely to report bloody diarrhea, however, less likely to get hospitalized compared to other age group. Age 10-59 had higher likelihood to acquire campylobacteriosis in foreign countries, and also the observed seasonality in summer was mainly driven by the cases in 10-59 years of age, implicating behavior factors contribute significantly for campylobacteriosis incidence in this age group. In elderly group, >60 years of age, significantly higher rate of hospitalization was observed in addition to clear increasing trend of *Campylobacter* incidence. Furthermore, geographical differences were observed in county level in Michigan: rural counties showed higher risk for campylobacteriosis than urban counties regardless of age. Within rural areas, age-specific risk factors were also observed, i.e. 10-19 years of age reported higher likelihood of contacting livestock than other age groups.

Based on our results, further studies are strongly warranted to investigate the agespecific risk factors, i.e. a case-control study with the residence (urban vs rural) matched, so effective preventive measures can be designed. Especially, morbidity and mortality in elderly population due to campylobacteriosis needs to be monitored. With the anticipated expansion of older ages (>65 years) in the future, it is strongly warranted to investigate the specific risk factors for this age group, so the morbidity and economic impact due to campylobacteriosis can be minimized. Ecological studies to investigate the association between the density of potential reservoirs like chicken and cattle and *Campylobacter* incidence in county level is also proper, given our result on the association between livestock contact and *Campylobacter* incidence in rural areas and previous literatures. Also, a moledular epidemiological study focusing in human patients residing in rural counties, especially in 10 counties that showed the highest incidence in Michigan, and the environment, including chickens, cattle and water, i.e. river, stream and well, will help understanding the transmission dynanimcs and the sources of campylobacteriosis in rural areas in Michigan.

By testing a subset of *C. jejuni* isolates (*n*=94) collected in 2011 and 2012, we observed a similar prevalence of fluoroquinolone and macrolide resistant *C. jejuni* from Michigan to what was reported for the nation in 2012. A high number of fluoroquinolone resistant *C. jejuni* infections were acquired from foreign countries. As 12.5% of the *Campylobacter* cases reported a history of foreign travel in Michigan in the descriptive epidemiology study, travel information may be crucial for implementing a proper treatment with antimicrobial agents. Furthermore, fluoroquinolone resistant *C. jejuni*, even without a selective pressure, warranting the need to further monitor the potential dissemination and evolution of the imported fluoroquinolone resistant *C. jejuni* strains in human population. We did not observe a significant association between fluoroquinolone resistant infection

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result may be mostly due to missing data and more importantly, improper study design for the research question. A large multi-center epidemiologic study, including different countries with varying prevalence of fluoroquinolone resistant *C. jejuni*, may be an appropriate approach. All cases with fluoroquinolone resistant *C. jejuni* infections may be matched with two or more susceptible infections based on age and sex. For the severity of disease, various outcomes should be evaluated, i.e. duration of illness, absent days at work, hospitalization status, length of hospital stays. Another approach can be made by investigating the pathogen. By using whole genome sequencing data, we could determine whether there is an association between carriage of certain virulence genes and resistance genes. Furthermore, we could conduct *in vitro* and *in vivo* study using human intestinal epithelial cell lines and animal models, respectively, to investigate whether fluoroquinolone resistant *C. jejuni* strains show higher colonization and invasion rates, and cause more severe colitis than fluoroquinolone susceptible *C. jejuni* strains.

A significantly higher tetracycline resistance was observed among human *C. jejuni* isolates in Michigan compared to the national report, and the resistance was significantly associated with contact with livestock, specifically cattle. A specific genotype, ST-982, was linked to both tetracycline resistance and livestock contact, implying that a tetracycline resistant, pathogenic clone may be circulating in the cattle population. Indeed, ST-982 was prevalent in cattle (*n*=22) and most of the isolates (86.4%) were resistant to tetracycline. Further supported by the PubMLST database, which shows that ST-982 has been only reported from cattle and farm environments in the U.S. previously, we conclude that cattle is an important reservoir for human tetracycline resistant *C. jejuni* infections in Michigan. Furthermore, in cattle study, we observed a marked difference in antimicrobial resistance

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profiles between farms. The differences were associated with specific genotypes, i.e. ST-459, which had a higher likelihood to be tetracycline resistant, were mostly observed in Farm C, while ST-1244 and ST-7679, which were linked with both fluoroquinolone-, tetracycline- resistance, were confined in Farm B and Farm A, respectively. We speculate that this could be due to the farm management factors, especially therapeutic and preventive antimicrobial treatments, forming a selective pressure, so the resistant clones are predominating in each farm. A molecular epidemiological study of *C. jejuni* isolates from a larger number of farms, including organic and conventional farms, in different part of Michigan can be conducted to investigate this further. The study should include not only cattle samples, but also the environmental samples, i.e. farm slurry, water, feed, animals coming in contact with cattle, to elucidate the possible transmission routes within and across herds in Michigan.

A total of eight STs, including ST-982, were recovered from both humans and cattle. Upon the phylogenetic analysis with human isolates from Michigan (*n*=35), we could see most of the shared STs were included in the clusters that were observed among cattle. This warrants a further study to elucidate the real genetic relatedness and evolutionary relationships of the isolates. We can start by conducting rep-PCR on the human isolates that shared the same STs with cattle. However, a more valid and useful approach is to use the whole genome sequencing data, i.e. single nucleotide polymorphism (SNP)/indel analysis or gene-by-gene approach, which may reveal the accurate genetic information in depth.

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Overall, we report a high frequency of campylobacteriosis in Michigan. With the increasing incidence and disease severity observed in this study, further studies, especially to identify the target specific risk factors, are strongly warranted. Furthermore, high prevalence of antimicrobial resistant *C. jejuni* found in this study warrants studies to further monitor the trend in human isolates and also investigate antimicrobial resistant *C. jejuni* in other potential sources, like chickens and environmental water in Michigan. Cattle was identified as an important reservoir for antimicrobial resistant *C. jejuni* infections in this study, warranting continuous monitoring and genetic investigation of the shared STs. Further study to elucidate the association between antimicrobial use in cattle farms and the antimicrobial resistance among circulating *C. jejuni* strains in cattle may help to draw another preventive measure for human antimicrobial resistant *C. jejuni* infections in