ENUMERATION, ISOLATION, AND DETECTION OF *CAMPYLOBACTER* SPECIES FROM FOOD STUFFS, FECAL SAMPLES, AND RIVER WATER OF EAST TENNESSEE

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"To expect the unexpected shows a thoroughly modern intellect." – Oscar Wilde

Dissertation Abstract

Campylobacter species (spp.) are a leading cause of bacterial foodborne illness. These Gram-negative, microaerophilic microorganisms are found within the intestinal tracks of warm-blooded animals and in humans can cause gastroenteritis, campylobacteriosis. Recent outbreaks are linked to raw milk, water, and puppies; however, these outbreak isolates have expressed antibiotic resistance, a main treatment line for campylobacteriosis. While *Campylobacter* spp. detection improves, cases are mainly sporadic and largely underreported. East Tennessee counties experience a higher prevalence of campylobacteriosis compared to other regions of the state based upon Tennessee Department of Health (TDH) surveillance. It is important for researchers to explore the presence of *Campylobacter* spp. in the environment to help elucidate the high incidence of campylobacteriosis. This dissertation aimed to identify if *Campylobacter* spp. existed within the East Tennessee geographic region.

The preliminary study collected samples (n=258) from local farmers' markets, parks, processing facilities, and rivers to better understand possible vehicles for campylobacteriosis within the region. Following incubation, presumptive positive samples (n=46) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Forty-two samples were confirmed as *Campylobacter* spp., twelve samples as *C. coli*, and two samples as *C. jejuni*. PCR confirmed isolates (n=39) were tested for antibiotic resistance. Eleven (28%) expressed resistance to antibiotics within the screening assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

A quantitative study collected samples (n=264) from four public access river sites to identify if *Campylobacter* spp. existed within river water throughout one calendar year within the Knoxville, Tennessee area. Following filtration and incubation, presumptive positives (n=244) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Confirmed isolates were identified as *Campylobacter* spp. (n=168) and *C. coli* (n=5). PCR confirmed samples (n=161) were tested for antibiotic resistance amongst a panel of eight antibiotics. Ninety-five (59%) isolates expressed resistance, according to CLSI guidelines.

By completing this study, a need to update campylobacteriosis risk factors, prevention education, and future treatment options has been identified, especially among TDH surveillance areas.

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Dissertation Organization

This dissertation is organized as follows.

Introduction lays out a brief overview of *Campylobacter*, its incidence, and the research conducted for this dissertation. Chapter I introduces *Campylobacter* in detail, provides a background on previous *Campylobacter* related outbreaks and research, and presents a need for additional *Campylobacter* research. Chapter II provides a brief *Campylobacter* review and information on previous *Campylobacter* related outbreaks and research involving produce, livestock animals, birds, and water. Additionally, Chapter II presents a preliminary study on the regional presence of *Campylobacter* species. Chapter III reviews *Campylobacter*, provides information on previous waterborne *Campylobacter* related outbreaks and research, and presents a study on the presence of *Campylobacter* species within regional waterways.

Introduction

Campylobacter species (*Campylobacter* spp.) are a leading cause of bacterial foodborne illness. *Campylobacter* spp. are Gram-negative, microaerophilic microorganisms found within the intestinal tracks of warm-blooded animals and can cause gastroenteritis in humans. Currently, there are 27 known species of *Campylobacter*, with three most commonly associated with campylobacteriosis in humans. Surveillance began in 1982 yielding 57 outbreaks from 1978 to 1986 (45 food, 11 water, and one unidentifiable) and 262 outbreaks from 1997 to 2008 (225 food, 24 water, 7 animal, and 6 unidentifiable). More recently, outbreaks have been linked to raw milk, contaminated water, and puppies; however, more importantly these outbreak isolates have expressed antibiotic resistance, a main treatment line for

campylobacteriosis. *Campylobacter* contain many virulence factors – secretion systems, a capsule, flagellum, cytolethal distending toxin (CDT), adhesions, and lipopolysaccharides (LPS) – that allow for survival in the environment. While *Campylobacter* spp. detection improves, cases are mainly sporadic and largely underreported with growing antibiotic resistance. East Tennessee counties experience a higher prevalence of campylobacteriosis compared to other regions of the state based upon Tennessee Department of Health (TDH) surveillance. It is important for researchers to explore the presence of *Campylobacter* spp. in the environment to help elucidate the high incidence of campylobacteriosis.

This study aimed to identify if *Campylobacter* spp. exist within food stuffs, animal feces, and river water from East Tennessee to better understand possible vehicles for campylobacteriosis within the region. From March through August 2017, samples (n=258) were collected from local farmers' markets, parks, processing facilities, and rivers. A food or fecal sample was diluted and incubated at 37˚C for 4 h then 44 h at 42˚C in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2). Water samples were vacuum filtered through a 0.45µm membrane filter. Samples were plated onto Campy CHROMagar® and incubated in a microaerophilic environment for 4 h at 37˚C then 44 h at 42˚C for isolation of *Campylobacter*. All presumptive positive samples (n=46) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Forty-two samples were confirmed as *Campylobacter* spp. – microgreens (n=5/5; 100%), sprouts (n=2/3; 66%), Tennessee River water (n=8/8; 100%), Hiwassee River water (n=6/7; 86%), cattle feces (n=5/6; 86%), swine feces (n=12/13; 92%), and geese feces (n=4/4; 100%). Twelve pork fecal samples were confirmed as *C. coli* and one

goose and one cattle fecal sample as *C. jejuni*. PCR confirmed isolates (n=39) were tested for antibiotic resistance. Twenty isolates expressed no growth and nineteen isolates were susceptible to antibiotics, 9 expressed intermediate conditions, and eleven expressed resistance to antibiotics within the screening assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The second part of this study aimed to better understand the spatiotemporal distribution of *Campylobacter* spp. and further elucidate surface water as a possible vehicle for campylobacteriosis by sampling multiple sites of the Tennessee River. Samples (n=264) were collected from four public access river sites throughout East Tennessee for one calendar year (November 2017-November 2018). Water samples were vacuum filtered through a 0.45µm membrane filter. Filters were plated onto Campy CHROMagar and incubated at 37˚C for 4h then 42˚C for 44h in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2). Presumptive positives (n=244) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Confirmed isolates were identified as *Campylobacter* spp. (n=168) and *C. coli* (n=5). All collection sites had relatively high levels of *Campylobacter* spp. isolated [site A (n=30/38; 79%), site B (n=29/38; 76%), site C (n=77/132; 58%), and site D (n=32/36; 89%)]. Five water samples were confirmed as *C. coli* from site D, which were obtained from January to March. PCR confirmed samples (n=161) were tested for antibiotic resistance amongst a panel of eight antibiotics. Ninety-five (59%) isolates expressed resistance, of which 23 (14%) isolates were resistant to one antibiotic, 27 (17%) isolates were resistant to two, 18 (11%) were resistant to three, 12 (7%) were resistant to four,

three (2%) were resistant to five, four (2%) were resistant to six, and five (3%) were resistant to seven, and three (2%) were resistant to all eight.

Chapter I: *Campylobacter:* A leading foodborne pathogen largely still unknown – A review

Abstract

Campylobacter species are a leading cause of bacterial foodborne illness. *Campylobacter* spp. are Gram-negative, microaerophilic organisms found within the intestinal tracks of warm-blooded animals and can cause gastroenteritis in humans. Originally classified as a *Vibrio* spp., there are 27 species of *Campylobacter* with three most commonly associated with campylobacteriosis. Surveillance began in 1982 yielding 57 outbreaks from 1978 to 1986 (45 food, 11 water, and one unidentifiable) and 262 outbreaks from 1997 to 2008 (225 food, 24 water, 7 animal, and 6 unidentifiable). More recently, outbreaks have been linked to raw milk, contaminated water, and puppies; however, more importantly these outbreak isolates have expressed antibiotic resistance, a main treatment line for campylobacteriosis. *Campylobacter* contain many virulence factors – secretion systems, a capsule, flagellum, cytolethal distending toxin (CDT), adhesions, and lipopolysaccharides (LPS) – that allow for survival in the environment. While *Campylobacter* spp. detection improves, cases are still majority sporadic and significantly underreported with growing antibiotic resistance. It is important for researchers to explore the presence of *Campylobacter* spp. in the environment to help elucidate the high incidence of campylobacteriosis.

Introduction

Campylobacter

Previously classified as *Vibrio* species and once known to be a cause of veterinary spontaneous abortions, *Campylobacter* species (spp) are microaerophilic, Gram-negative, curved rods with a single, polar flagellum *(17, 46)*. In the 1950s, *Campylobacter* spp. were first identified from human blood cultures, which researchers believed to be uncommon, opportunistic pathogens. These early detected pathogenic microorganisms were classified as *Vibrio fetus* and related *Vibrio (46)*.

In 1978, Bennington, Vermont had one-fifth (n=2000) of its community suffering from abdominal pain or cramps and diarrhea, followed by malaise, headache, and fever. Consumption of the municipality's water was found to be significantly correlated with illness ($p \leq 0.005$). Epidemiologists identified that, while the city chlorinated its main water supply, it was not filtered. Additionally, while the water line in use required chlorine, collected water samples did not show residual chlorine. Of nine cases sent for testing, five had positive rectal swabs cultured at the Centers for Disease Control and Prevention (CDC) for *Campylobacter fetus*. This resulted in the first known *Campylobacter* spp. outbreak in the United States *(48)*.

Campylobacter spp. are naturally occurring microorganisms within the normal intestinal microflora of warm-blooded animals and are commonly associated with birds allowing for easy entrance into the environment by defecation of these hosts *(2)*. They are a leading cause of an acute diarrheal illness known as campylobacteriosis, which can lead to abdominal pain, cramps, diarrhea, malaise, headache, or fever. In the most severe cases, bloody stool mirroring ulcerative colitis might be experienced *(17, 57)*. Typically, symptoms are expressed 24-48 hours after consumption, but this varies

based on many factors, such as immune status, age, and number of organisms consumed *(21)*. Symptoms can last from 2-7 days, but a person might shed the microorganism for months following the cessation of symptoms *(17)*.

There are an estimated 9.4 million cases of foodborne illness, in the U.S., from 31 known pathogens annually, which result in 55,961 hospitalizations and 1,351 deaths, yet most foodborne illnesses are not detected or reported to health officials *(38)*. In 2016, *Campylobacter* had the highest incidence of confirmed cases with 11.79 cases per 100,000 persons and 17.43 culture-independent diagnostic tests (CIDTs) confirmed cases per 100,000 persons according to the annual Foodborne Diseases Active Surveillance Network (FoodNet) progress report *(27)*. However, FoodNet reports 99% of all *Campylobacter* cases are sporadic meaning they are not recognized to have an epidemiological link to an outbreak *(47)*.

Campylobacter species

In 1963, Sebald and Veron suggested a new genus, *Campylobacter*, for these *Vibrio*-like organisms leading to the change of *Vibrio fetus (V. fetus) and Vibrio bubulus (V. bubulus)* to *Campylobacter fetus (C. fetus)* and *Campylobacter bubulus (C. bubulus)*. In the mid 1970's, researchers advocated for four species – *fetus, coli, jejuni,* and *sputorum (51)*. *Fetus* represented the type of species, *coli* was for swine isolates, and *jejuni* for cattle, human, or sheep isolates. *Sputorum* was divided into two subspecies – *sputorum* and *bubulus (33)*. *Sputorum* represented isolates collected from the sputum first seen in a bronchitis patient *(36, 49)* and *bubulus* those from "vaginal and preputial secretions, sperm, fetuses, and fetal membranes" *(51)*. As research and interest grew, the number of *Campylobacter* spp. and detection methodologies became established.

Currently, there are 27 species (Table 1.1) and eight subspecies of *Campylobacter* with *Campylobacter jejuni (C. jejuni)* serving as the species of greatest concern causing most of the campylobacteriosis cases worldwide *(17, 26, 33)*.

While not the main cause of campylobacteriosis, *C. fetus* was the first species to be identified. *C. fetus* subsp. *fetus* was initially associated with spontaneous abortions in sheep and cattle *(29)*. *C. fetus* subsp. *venerealis* was associated with bovine genital campylobacteriosis leading to infertility, abortion, and embryonic death. The two species which cause the majority of foodborne illnesses are *C. jejuni* and *C. coli*. Originally, these two species were isolated from the stool of cattle and pigs. They differ in the hydrolysis of sodium hippurate, for which *C. jejuni* are positive *(12, 40)*. Another species of concern for humans, *C. lari*, are associated with human bacteremia and diarrhea, especially in immune-deficient patients. Key phenotypic differences amongst *C. lari*, *C. jejuni* and *C. coli* are 1) an inability to hydrolyze indoxyl acetate and 2) nalidixic acid resistance *(18, 33, 37)*. Currently, any *Campylobacter* strain that can produce urease, is susceptible to nalidixic acid, or can both produce urease and is susceptible to nalidixic acid are considered *C. lari (33, 37)*.

In addition to pathogenic *Campylobacter* spp., there are some species found naturally occurring within the human oral cavity. These species are as follows: *C. concisus, C. carvus, C. showae, C. rectus, and C. gracilis.* Tanner et al. *(45)* studied multiple *C. rectus*, *C. concisus*, and *C. gracilis* strains extracted from periodontal pockets to understand characteristic similarities and differences. Researchers concluded that while similar, *C. rectus* have a smaller molecular weight than *C. concisus* and *C. gracilis (45)*. *C. ureolyticus* formerly *Bacteroides ureolyticus* have been

extracted from genital, dental, and soft-tissue sites *(19)*. *C. gracilis* formerly *Bacteroides gracilis* have been associated with head, neck, and lung infections *(19)*. *C. upsaliensis* have been found in bacteremic humans, human abscesses, and abortion cases *(22, 33)*. While *C. hominis* have been detected in healthy humans, diarrheic humans, and otherwise healthy humans, can also test positive for *C. lanienae*, if they work in meat processing facilities since they are associated with seemingly healthy swine *(22, 25, 33)*.

A Snapshot of USA Campylobacter Outbreaks and History of Detection

Epidemiologist considered *Campylobacter* spp. to be the primary cause of abortions in animals without any links to impact on public health until the 1978 outbreak in Bennington, VT. Less than one month later, a small outbreak in Colorado linked to *C. fetus* subsp. *jejuni* occurred within a farming family. Of the five family members, three experienced malaise, muscle pain, and nausea with one family member experiencing bloody diarrhea *(4)*. Cases were treated with erythromycin and stool cultures were collected from cases, non-ill family members, and farm animals (chickens, swine, sheep, calves, and a cow). Fecal samples from non-ill family members, chickens, swine, sheep, and calves were negative; however, fecal samples from all cases and the cow were positive for *C. fetus* subsp. *jejuni*. It was identified that all three cases reported drinking raw cow's milk *(4)*.

In 1982, laboratory surveillance of *Campylobacter* began in 11 states which already had surveillance programs for *Salmonella* and *Shigella.* As states began *Campylobacter* surveillance, the national incidence rate increased; however, the incidence rate remained proportional to the national population, meaning the increase was caused by new surveillance and not an increase in cases. Through surveillance, 57

outbreaks were identified from 1978-1986 involving *Campylobacter* spp. related to water (n=11), food (n=45), and one unidentified (n=1) *(46)*. This yielded 41,343 isolates (n=3,630 non-reported) with 99% (n=37,478) of outbreaks due to *C. jejuni* followed by *C. fetus* (0.4%)*, C. coli* (0.2%)*, C. laridis* (0.02%)*, C. sputorium* (0.003%)*, C. faecalis* (0.003%)*, and a Campylobacter*-like organism (0.003%) *(46).* At that time, researchers saw a bimodal peak of illness in May and October, with difficulty isolating *Campylobacter* spp. in warmer months from environmental samples *(46)*.

Taylor et al. *(47)* evaluated common source outbreaks from 1997 to 2008, in which *Campylobacter* spp. were the causative agent. Researchers reviewed a total of 262 outbreaks, 9,135 illnesses, 159 hospitalizations, and three deaths related to campylobacteriosis. As a nation, the United States averaged 16 outbreaks annually from 1997-2002, increasing to 28 from 2003-2008, with the majority occurring in warmer months. Taylor et al. *(47)* reported that while raw milk had been historically the most common outbreak source, two of the three deaths were related to a single outbreak of contaminated water. Of the 262 reported outbreaks, dairy products were linked to 65 outbreaks (24.8%), followed by poultry (n=25 or 9.5%), and produce (n=12 or 4.6%). Additionally, the majority of *Campylobacter* outbreaks occurred during summer months with 128 occurring from May-August of 2005-2008. The three largest outbreaks were reported during warmer months, one foodborne (n=1,644) and two waterborne (n=1,450, n=781) *(23)*. While raw milk and poultry are a primary focus when evaluating risk of *Campylobacter*, water is a routinely identified vehicle either causing illness through interaction with contaminated potable or recreational water. In 2017, *C. jejuni* was associated with an outbreak attributed to municipal water which was contaminated

from an adjacent concentrated animal feeding operation (CAFO) runoff into the area surrounding two wells that supplied water to the city *(34)*.

The CDC reviewed outbreaks related to unpasteurized or raw milk from 2007-2012. Over six years, a reported 81 outbreaks were reported from 26 states that resulted in 979 illnesses, 73 hospitalizations, and zero deaths *(32)*. Determining a single causative agent is not always feasible, but 78 of these 81 outbreaks had an identifiable etiological pathogen. The CDC determined that 81% (n=62) of these outbreaks were caused by *Campylobacter* spp. *(32)*. The US averaged 3.3 raw milk outbreaks from 1993-2006; however, from 2007-2012, the US averaged 13.5 outbreaks *(32)*. One of those occurred in 2011, when Alaskan public health officials identified a cluster of matching pulsed-field gel electrophoresis (PFGE) *Campylobacter* isolates. Following an investigation, seven residents were identified as consuming raw milk from a local herd share program in the Matanuska-Susitna Valley *(41)*. Additionally, in 2013, there was a large-scale outbreak within the Kenai Peninsula of Alaska due to raw milk consumption. Epidemiologists identified over 30 cases ranging from 7 months of age to 72 years old testing positive for *C. coli* infection due to a herd share program. Two cases were hospitalized and four developed reactive arthritis *(42)*. In 2014, there were two separate outbreaks related to raw milk consumption. The first occurred in Utah, where the sale of raw milk is legal with a Utah Department of Agriculture and Food permit. Following routine monthly testing, the dairy's products tested within the acceptable levels. It was not until "enhanced testing procedures recovered *C. jejuni*" following a cluster of campylobacteriosis cases reported to the health department *(11)*. From May to November 2014, ninety-nine cases were identified, and the dairy's permit was revoked

(11). The second raw milk outbreak occurred in Wisconsin following a team dinner where raw milk was served as a beverage. Wisconsin health officials tested fecal samples from the dairy, which tested positive to the same *Campylobacter* strain in the 22 cases *(35)*.

Campylobacter spp. are rarely identified as a biological hazard for fruits and vegetables. However, there have been some well documented outbreaks associated with produce, demonstrating the ability of *Campylobacter* spp. to survive within this environment *(8)*. In a recently completed meta-analysis of *Campylobacter* spp. prevalence in fresh fruits and vegetables, the prevalence of contamination was estimated to be 0.53% amongst fresh produce *(30)*. Bean and sprouts were found to have the highest reported prevalence (11.08%) *(30)*.

Doyle and Schoeni *(12)* identified that while rare, fresh produce, specifically ones packaged in a polyvinyl chloride film-wrap can serve as a vehicle for campylobacteriosis. They found *C. jejuni* contamination within 1.5% (n=200) of retail mushrooms; however, contamination was not likely caused by compost due to its heat treatment. Their next hypothesis was the peat and lime casing material in addition to the moist growth environment aided in the survival of *C. jejuni.* These wrapped mushrooms, while held at refrigeration temperatures, are packaged in a low oxygen environment, which allows for the survival of this microaerophilic pathogen *(12)*.

While the vast majority of outbreaks are linked to milk and poultry products, increasing evidence can be found that *Campylobacter* spp. may have greater tolerance to the environment than first thought, considering it shows little tolerance to common environmental stressors. When evaluating the adaption of *C. jejuni* to the phyllosphere

and rhizosphere of radishes and spinach, populations declined in all temperatures, but cooler temperatures of 10ºC and 16ºC allowed for survival up to 28 days in the rhizosphere of these crops *(6)*. The ability to survive in the soil and water for increased periods of time may help persistence and spread to typical warm-blooded hosts.

In recent years, outbreaks have been related to a variety of sources ranging from raw milk to contaminated water at an obstacle course race. In 2012, twenty-two cases were identified as having a *C. coli* infection following participation in a long-distance obstacle adventure race in Nevada. Epidemiologists were able to identify a strong association (OR=19.4; $p<0.001$) between the consumption of mud while competing in the race and campylobacteriosis *(58)*. This outbreak brought awareness to *Campylobacter* spp. capability to survive in low-oxygen environments without a warm-blooded host, which could further explain sporadic cases or those without an epidemiologic link to a known outbreak.

Although significant decreases in the prevalence of many pathogens have been observed over time, incidence rates of *Campylobacter* spp. associated-infections have continued to climb. There was a 12% increase in the incidence rate of campylobacteriosis for 2018 when compared to 2015-2017 FoodNet data *(44)*. This points to the continued need to investigate and uncover sources of contamination and methods for improved control within food systems.

Campylobacteriosis Treatment and Sequelae

Traditionally, campylobacteriosis is treated with fluids or oral antibiotics. Antibiotics are classified into over 20 classes to which each individual antibiotic is identified as either bactericidal or bacteriostatic. Bactericidal antibiotics kill the bacteria, while bacteriostatic antibiotics halt the growth and allow the immune system time to

attack and inactivate the bacteria. Antibiotics are also classified by their mechanism of action: cell wall synthesis, protein synthesis, DNA synthesis, RNA synthesis, folate synthesis, or combinations. Penicillin and cephalosporin classes inhibit bacterial cell wall synthesis by inhibiting transpeptidase. Macrolides and tetracyclines are bacteriostatic, while aminoglycosides are bactericidal; however, these three classes all inhibit protein synthesis. Fluoro/quinolones block DNA synthesis by binding DNA gyrase *(52)*. Campylobacteriosis is commonly treated with erythromycin (a macrolide), tetracycline, or ciprofloxacin, which is a fluoro/quinolone *(39)*.

As *Campylobacter* spp. continue to be a leading cause of bacterial foodborne illness, researchers continue to investigate antibiotic resistance and sensitivity. In 1999, researchers in Minnesota reviewed *Campylobacter* isolates submitted to the state laboratory from 1992 to 1998. These years were investigated since fluoro/quinolones were not approved for use within the poultry industry until 1995 and investigators aimed to identify if there was a change in quinolone- or nalidixic acid-resistant isolates after the release of this antibiotic class into the poultry industry. Researchers found there was an increase in quinolone-resistant isolates from 1.3% to 10.2%; however, most cases were linked to international travel or taking quinolones preventatively while traveling or upon return. Similarly, researchers in Spain, Taiwan, and the United Kingdom found an increase in quinolone-resistant *Campylobacter* isolates following the release of fluoroquinolones for use within the poultry industry *(39)*.

In 1996, the CDC began the National Antimicrobial Resistance Monitoring System-Enteric Bacteria (NARMS) and in 2003 it went nationwide. NARMS aims to monitor “antimicrobial resistance among enteric bacteria from three sources: humans,

retail meats, and food animals" *(10)*. Since 2016, several noteworthy outbreaks associated with *Campylobacter* spp. exhibiting resistance to multiple antibiotics have occurred.

The first occurred in early August until October of 2016 due to the consumption of raw milk in Colorado. While raw milk sales are illegal in Colorado, herd share programs are permitted. Due to a lack of assistance by the herd share program, state public health officials issued a notice leading to the participation of 91 shareholder households in a follow-up interview. From interviews, twelve confirmed cases and five probable cases were identified ranging in age from 12-68 years yielding one hospitalization and zero deaths. PFGE was available for ten cases and four milk samples for *C. jejuni*. All PFGE confirmed case isolates and two milk isolates were sent to NARMS for antimicrobial testing. NARMS tested five isolates, of which all were ciprofloxacin-, tetracycline-, and nalidixic acid-resistant *(7)*.

The second reached national news in 2017 when an outbreak of *Campylobacter* related to puppies found at pet stores reached 18 states and had a total of 118 cases. During this outbreak, 105 cases reported a dog exposure of which 95% reported contact with a puppy from a pet store and eight reported contact or purchasing a puppy from an additional pet store. While less than 25% of cases (n=26) were hospitalized and there were no identified deaths, this outbreak brought attention to a notable level of antibiotic treatment within the commercial dog industry for two reasons: (1) types of antibiotics used and (2) widespread antibiotic resistance. After a review of 149 pet store puppy records, puppies received prophylactic treatment (n=78), antibacterial treatment (n=2), or both prophylactic and antibacterial treatment (n=54). Broad spectrum antibiotics,

such as doxycycline and azithromycin, accounted for 81% of dispensed antibiotics in addition to the use of veterinary antibiotics, such as sulfadimethoxine.

Human (n=45) and puppy (n=11) *C. jejuni* isolates were tested via whole-genome multilocus sequence typing. Three clades were found with two containing ≤32 genetically different alleles and one with ≤30 genetically different alleles. There were 18 isolates matched to all three clades that were additionally tested. All eighteen isolates were resistant to the following antibiotics: azithromycin, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, telithromycin, tetracycline, and some samples resistant to gentamicin and florfenicol *(31)*. These antibiotics represent the main treatment lines for patients, clearly impacting efficacy of any medical interventions *(9)*. As antibiotic resistance rises, cases can experience longer disease duration or treatment complications without the invention of newer antibiotics, which can result in economic losses and potential health effects from post-infectious secondary illnesses or sequelae.

Campylobacteriosis sequelae

Following campylobacteriosis, individuals are known to experience various sequelae, including Guillain-Barré syndrome (GBS), reactive arthritis (ReA) or Reiter's Syndrome, and irritable bowel syndrome (IBS). The World Health Organization (WHO) reports one-third of GBS cases are associated with campylobacteriosis and another third of patients will develop GBS within two years *(53)***.** GBS is a neurological disorder caused by the immune system attacking the peripheral nervous system leading to weakness, an inability to breathe, and paralysis. Most cases recover with some experiencing varying degrees of weakness. Currently, acute and rehabilitative therapies are available for GBS cases; however, in 2013, van den Berg et al. *(50)* reported 3-7%

death due to GBS complications *(53)*. Unlike GBS, ReA and IBS have a less formal diagnostic process and are still being fully understood.

Reactive arthritis (ReA) or Reiter's Syndrome is an acute arthritis triggered by a gut, genital, or urinary tract infection *(15, 16)*. In 1981, Gumpel et al. *(15)* identified seventy-seven *Campylobacter* positive human stool samples outside London, England. Of these 77 cases, 47 were hospitalized with 33 of the hospitalized cases being 16 years of age or older. After reviewing cases over 16 years of age, eight were identified as experiencing a possible ReA episode and two sought medical treatment. Of these two cases, one was a 30-year-old woman with a swollen wrist, two swollen knees, a tender toe joint, and an arthritic neck. All of her symptoms followed an episode of excessive watery diarrhea *(15)*. Her condition resolved itself over a 6-month timeframe and she tested negative for HLA B27, an antigen believed to be involved in ReA. The second involved a middle-aged man with a swollen knee following a 6-week spell of diarrhea and cramping. While widespread calcium crystals were found on his joints, his synovial fluid was sterile *(15)*. This study aimed to estimate the frequency of ReA associated with positive *Campylobacter* spp. diarrhea due to an assumption of ReA and campylobacteriosis being an additional cause of ReA, similar to salmonellosis *(15)*. Hannu et al. *(16)* evaluated *Campylobacter* positive cases from April 1997 to September 1998 in Finland. Cases were matched by age, sex, and location to controls to identify arthritis symptoms after campylobacteriosis. A total of 405 matched pairs were interviewed with 38% (n=220 of 582) *Campylobacter* positive cases reporting ReA versus *Campylobacter* negative controls reporting 24% (n=185 of 758). Of the 220 ReA controls, 113 were clinically examined of which 45 were diagnosed as experiencing

ReA. Similar to Gumpel et al. *(15)*, cases in this study most commonly reported inflammation of the knees *(16)*,

IBS or “a chronic, episodic medical condition associated with abdominal pain or discomfort and altered bowel habits,” is estimated to develop in 4-32% of bacterial gastroenteritis cases *(13)*. In a 2013 study by Sung et al. *(43)*, rats were injected with 100 million *C. jejuni* cells in three populations – juvenile only, adult only, or both juvenile and adult rats. Following incubation, researchers identified rats with a previous injection of *C. jejuni* recovered quicker than those who had not. Adult rats had a shorter duration than juvenile ones, and most juvenile rats had cleared an infection by adulthood. Additionally, researchers noticed small intestinal bacterial overgrowth (SIBO), which is assumed to be a contributing symptom of IBS. Of the rats (n=49) infected as both juvenile and adult, 47% developed SIBO while only 26% of adult only (n=50) infected rats developed SIBO. Results from this study reflected previous epidemiologic findings that immunity can be acquired, and post-infection IBS can develop following repeated infections *(43)*.

Campylobacter virulence factors

Pathogenic microorganisms acquire virulence factors as a mechanism of protection against a host’s immune system or for survival within a new environment in order to cause infection within another host. *Campylobacter* spp. are competent pathogens meaning they have the ability to uptake DNA from their environment by *in vitro* or host colonization *(55)*. *Campylobacter* spp. DNA uptake is driven by the use of carbon dioxide and bacterial cell density *(55)*. *C. jejuni* use transposon mutagenesis or the ability to transfer genes to the host’s chromosomes to enter competence.

Campylobacter spp. have the following virulence factors: secretion systems, capsule, flagellum, toxin, adhesions, and lipopolysaccharides *(55)*.

Secretion Systems

Campylobacter spp. secretion systems are still being researched, but the role of protein CiaB has been elucidated. CiaB allows for *Campylobacter* spp. to affectively invade host epithelial cells. During a chicken study, mutant strains were not capable of host colonization allowing researchers to better understand its importance. *Campylobacter* spp. express a Type III secretion system utilizing CiaB proteins through the flagella by excreting itself as FlaC. These proteins are necessary following host cell contact allowing the Type III secretion needle to inject pathogenic material into the host cell *(21, 52)*. Additionally, *Campylobacter* spp. are capable of moving proteins from the inner membrane to the outer membrane by the flagella allowing for infection of additional cells, mimicking a Type II and Type IV secretion system *(55)*. Recent research by Bleukmik-Pluym et al. *(5)* identified a Type VI secretion system within certain *C. jejuni* strains due to the presence of T6SS genes, *hcp* and *vgrG,* following whole genome sequencing. By expressing a working T6SS, *Campylobacter* have the ability to attached and infect host cells and survive in the presence of bile salts, which aim to eliminate waste from the body *(24)*.

Capsule

Campylobacter spp. express a capsule, which allows for intestinal cell adherence and invasion, prolonged infection *in vivo* during mouse studies, and resistance to human serums *(28, 55)*. Capsule composition varies by species, but it contains sugars, like heptose and xylose, not traditionally associated with bacterial capsules. Traditionally, bacterial capsules are comprised of glycoproteins, polypeptides, and D-

glutamic acids. Researchers attribute capsule variety to phase variation instead of species mutations *(55)*.

Flagellum

Campylobacter spp. express a single, polar flagellum, which is used for the colonization and invasion of host cells and movement allowing the pathogen to survive *(14, 55)*. The flagellum is comprised of a basal body, hook, and filament *(20)*. The filament contains FlaA, a major flagellin, and FlaB, a minor flagellin *(14, 20)*. Flagella with mutated *flaA* expressed decreased motility, while mutated *flaB* did not express a significant decrease in motility *(14)*. In a study by Black et al. *(3)*, adults (n=111) were infected with various dosages of *C. jejuni* strains to better understand motility. Researchers found that while all collected fecal samples contained leukocytes, only those infected with motile strains were identified within fecal samples *(3)*. The flagella have no secretion systems, but utilize three secretion proteins, Cia, FlaC, and FspA. Cia is found in three *Campylobacter* spp. (*C. coli, C. upsaliensis,* and *C. lari*) and require a basic flagella structure to aid in motility and host cell invasion *(14)*. FlaC protein is not a part of the filament nor required for motility but can extend as far as the hook portion *(14)*. The final secretion protein, FspA, is specific to *C. jejuni* and has multiple forms, but only FspA2 is “toxic to eukaryotic cells” *(14)*. *Campylobacter* flagella have toll-like receptor 5 (TRL5). TLR5 recognizes the amino and carobxy conserved regions of the flagellins, which prevents host cell innate immune system response. Finally, *Campylobacter* flagella are glycosylated, which was once believed to only occur in eukaryotes; however, *C. jejuni* are incapable of building a flagellum if the filament is not glycosylated *(14)*. Glycosylated surface structures allow for protection against immune system response by utilizing antigenic variation *(21)*.

Cytolethal Distending Toxin (CDT)

Cytolethal distending toxin is made up of three proteins, which form a complex – CdtA, CdtB, and Cdt – used to take up host cells leading to cell death by preventing cell division *(1, 55)*. CdtA and CdtC are structurally similar and bind HeLa cells causing endocytosis of ricin allowing CdtB to perform. CdtB performs similar to a DNAse by colonizing the host cell's nucleus leading to DNA damage and the signaling of DNA-repair proteins *(1, 55)*. In a mouse model using *Helicobacter hepaticus* and *C. jejuni*, wild-type mice were colonized by CDT. Mutant-mice without a nuclear-factor kappa-chain-enhancer of activated B cells (NF-ϰB) could not be successfully colonized, similarly this was found in a chicken. This study identified the importance of NF-ϰB to the functionality of CdtB *(55)*.

Adhesions

Surface adhesions or pili are used to successfully colonize a host cell *(55)*. *Campylobacter* spp. do not have any known pili; however, they do have Type-II like secretions systems similar to *Vibrio cholera*, which allows for pilus assembly. In addition to a Type-II like secretion system, many adhesion proteins are also found, like *CadF, JlpA, Cap A, Peb1*, and Cj1496c *(55)*. *CadF* is essential for binding and host cell invasion *(55)*. *JlpA* and *CapA* are lipoproteins found within *C. jejuni*. *JlpA* creates the inflammatory responses experienced during campylobacteriosis and *CapA* acts as an autotransporter. In a chicken study, *CapA* was identified when mutant strains were not capable to adhere to cells, leading to a decrease in colonization and infection *(55)*. *Peb1 (CBF1)*, located in the periplasm, acts like an ATP-binding cassette transporter allowing high levels of aspartate and glutamate binding. In studies, *peb1* mutant strains could not affectively grow and cause infection; however, it was unclear if poor infection

occurred due to the knockout of *peb1*, low amino acid levels, or both *(55)*. The final adhesion has been identified as Cj1496c for effective host cell infection for *C. jejuni* infections specifically. Cj1496c is a periplasmic magnesium transporter protein and important to the strain's ability to adhere to host cells. This is the only known mechanism *(55)*.

Lipopolysaccharides (LPS)

Lipopolysaccharides are found extensively on the surface of Gram-negative microorganisms. The LPS is made of 3-layers – Lipid A, core, O-antigen – which allow for adhesion and surface alteration to avoid host immune response *(2, 55)*. It is believed that the LPS is the primary factor to GBS. Sialic acid and N-acetyl neuraminic acid are uncommon within prokaryotes. When sialic acid is paired to D-galatosidase, it appears to mimic a ganglioside or a molecule on the cell's surface involved in host cell function and expressed in nervous tissues *(2, 54)*. Previous studies identified GM_1, a ganglioside, as having correlation to the presence of IgM antibodies; however, GM_1 was not present within all GBS positive cases. When Yuki *(56)* studied GBS+/Cj+ (GBS positive/*C. jejuni* positive) patients and GBS-/Cj+ (GBS negative/*C. jejuni* positive) patients, he identified similar results. Yuki found GBS+/Cj+ cases with IgG anti-GM_1 antibodies and no anti-GM_1 antibodies identified within GBS-/Cj+ cases *(56)*. Yuki further extracted the LPS from GBS+/Cj+ collected samples for Penner's 16 serotype (PEN 19). The LPS contained "galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid," which make up the composition of GM_1 *(56)*. This showed "molecular mimicry between nerve tissue and the infectious agent isolated from a GBS patient" *(56)*.

Conclusion

Since first being identified as a foodborne pathogen in the 1980s, there remains a lack of understanding as to where *Campylobacter* spp. are located within the natural environment given their fastidious nature. Even as our ability to detect this organism improves, cases of campylobacteriosis continue to be predominantly sporadic and underreported deterring from epidemiologic studies for source identification. Additionally, with increased surveillance and infections being increasingly identified via culture independent tests, antibiotic resistance knowledge is essential to understand treatment of campylobacteriosis in the future. Therefore, it is important to better understand the prevalence of *Campylobacter* spp. within the environment, mechanisms of adaptation, and the potential to contaminate vulnerable commodities such as produce, which have been linked but not traditionally focused on as vehicles for *Campylobacter* spp. infections.

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Chapter II: Enumeration, Isolation, and Detection of *Campylobacter* species within the Environment of East Tennessee

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Abstract

Campylobacter species are among the main causes of bacterial gastroenteritis in the United States and commonly associated with the consumption of poultry, meat, and raw milk. East Tennessee counties experience a higher prevalence of campylobacteriosis compared to other regions of the state, based upon Tennessee Department of Health (TDH) surveillance. This study aimed to identify if *Campylobacter* spp. exist within food stuffs, animal feces, and river water from East Tennessee to better understand possible vehicles for campylobacteriosis within the region. From March through August 2017, samples (n=258) were collected from local farmers' markets, parks, processing facilities, and rivers. A food or fecal sample was diluted and incubated at 37˚C for 4 h then 44 h at 42˚C in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2). Water samples were vacuum filtered through a 0.45µm membrane filter. Samples were plated onto Campy CHROMagar® and incubated in a microaerophilic environment for 4 h at 37˚C then 44 h at 42˚C for isolation of *Campylobacter*. All presumptive positive samples (n=46) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Forty-two samples were confirmed as *Campylobacter* spp. – microgreens (n=5/5; 100%), sprouts (n=2/3; 66%), Tennessee River water (n=8/8; 100%), Hiwassee River water (n=6/7; 86%), cattle feces (n=5/6; 86%), swine feces (n=12/13; 92%), and geese feces (n=4/4; 100%). Twelve pork fecal samples were confirmed as *C. coli* and one goose and one cattle fecal sample as *C. jejuni*. PCR confirmed isolates (n=39) were tested for antibiotic resistance. Twenty isolates (51%) expressed no growth and nineteen isolates (19%) were susceptible to antibiotics, 9 (23%) expressed intermediate growth, and eleven (28%)

expressed complete resistance to antibiotics within the screening assay, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. These data indicate that antibiotic resistant *Campylobacter* spp. are present in surface waters, animal feces, and sprouted seeds within East Tennessee and could result in negative implications for treatment and overall health complications for the region.

Introduction

Overview

Campylobacter species (*Campylobacter* spp.) are microaerophilic, Gram-negative, curved rods with a single, polar flagellum *(12, 33)*. First identified as a cause of veterinary spontaneous abortions, *Campylobacter* spp. are naturally occurring microorganisms within the normal intestinal microflora of warm-blooded animals *(1, 33)*. Campylobacteriosis is the diarrheal illness caused 24-48 hours following consumption, but this varies based on immune status, age, and number of pathogens consumed. A person might experience abdominal pain, cramps, diarrhea, malaise, headache, or fever for 2-7 days; however, one can shed the microorganism for months following the cessation of symptoms *(12)*. Campylobacteriosis can lead to post-infection sequelae, including Guillain-Barré syndrome (GBS), reactive arthritis (ReA) or Reiter's Syndrome, and irritable bowel syndrome (IBS). The 2016 Foodborne Disease Active Surveillance Network (FoodNet) progress report reported *Campylobacter* as having the highest incidence of confirmed cases at 11.79 cases per 100,000 with the majority (99%) of *Campylobacter* cases occurring sporadically with peaks during the warmer months of May-August *(16, 34)*.

Reservoirs

Originally classified as *Vibrio* species, there are 27 identified *Campylobacter* spp. and eight subspecies with *Campylobacter jejuni* (*C. jejuni*) serving as the species causing the greatest number of human campylobacteriosis cases *(12, 15, 20)*. Since the surveillance of *Campylobacter* spp. began in 1982, *Campylobacter* spp. have been linked to outbreaks related to water and food *(9, 11, 21, 29, 30, 33, 34)*. The Centers for Disease Control and Prevention (CDC) educates consumers on increased risks of

campylobacteriosis due to the consumption of raw or undercooked poultry, cross contamination with raw or undercooked poultry, raw dairy products, untreated water, and produce *(6)*. In a 2002 review by Newell *(19)*, *Campylobacter* spp. are considered commensals in birds and have the ability to colonize the gut of most mammals. Gut colonization, in birds, appears within 2-3 weeks after hatch and can be detected among entire flock within 3 days *(19)*. Countries with poultry flock surveys report up to 95% of flocks test positive for *Campylobacter* spp. *(19)*.

Campylobacter and the Environment

Following a three-year study of a southern Ontario, Canada watershed, thermophilic *Campylobacter* spp. were identified in 57-79% of the samples collected from five locations using quantitative PCR *(37)*. Biweekly samples from five sites upstream from municipal drinking water treatment plants were collected by removing water 2-3 m from the river's edge and 10-20 cm below the surface in fast flowing areas from 2005 through 2008. In 2007, waterfowl fecal samples were collected from areas near collection sites. Researchers isolated *Campylobacter lari* (66%), *C. jejuni* (30%), and *C. coli* (9%) from river water positive samples *(37)*. *C. jejuni* (73%), *C. lari* (27%), and *C. coli* (13%) were isolated from waterfowl fecal samples *(37)*. High quantities of nalidixic acid susceptible *C. lari* were identified among both river water and fecal waterfowl samples. Following 16S rRNA sequence, 100% homology was identified among a seagull fecal sample and a nearby collected water samples *(37)*. Research from this study allows for a better understanding of surface and drinking water contamination in southern Ontario and how they may serve as modes of transmission for campylobacteriosis.

Campylobacter *and Produce*

Campylobacter spp. are rarely identified as a biological hazard for fruits and vegetables. However, there have been well documented outbreaks associated with produce, demonstrating the ability of *Campylobacter* spp. to survive within this environment *(4)*. In a recently completed meta-analysis of the prevalence of *Campylobacter* spp. in fresh fruits and vegetables, the prevalence of contamination was estimated to be 0.53% among fresh produce *(17)*. Bean and sprouts were found to have the highest reported prevalence (11.08%) of fresh produce *(17)*.

Doyle and Schoeni *(10)* identified that while rare, fresh produce, specifically ones packaged in “polyvinyl chloride film-wrap” can serve as a vehicle for campylobacteriosis. *C. jejuni* contamination was identified within 1.5% (n=200) of retail mushrooms. Their hypothesis was the peat and lime casing material in addition to the moist growth environment aided in the survival of *C. jejuni.* These wrapped mushrooms, while held at refrigeration temperatures, are in a low oxygen environment, allowing for the survival of this microaerophilic pathogen *(10)*.

To better understand the Danish national incidence, researchers led a prospective case-control study from January to December of 2016 utilizing an online questionnaire, resulting in 887 cases and 2915 controls *(13)*. Researchers aimed to determine sources including non-food items that place 1 to 30 year olds at risk for campylobacteriosis. Overall, traveling abroad was the main risk factor for campylobacteriosis (OR=4.6, 95% CI 3.7-5.7); however, there were many additional risk factors found to be statistically significant, including those who bathed in fresh water (OR=6.0, 95% CI 3.0-11.9) or those who consumed whole chicken (OR=2.2, 95% CI 1.8-2.9), boneless chicken fillets (OR=2.1, 95% CI 1.6-2.6), or chicken thighs (OR=1.6,

95% CI 1.2-2.0) *(13)*. Additional foods, such as fruits and vegetables were included for their association with campylobacteriosis. Campylobacteriosis was determined likely following the consumption of fresh strawberries (OR=1.6, 95% CI 1.3-2.1), fresh raspberries (OR=1.5, 95% CI 1.1-1.9), fresh blueberries (OR=1.2, 95% CI 1.1-1.8), and frozen berries (OR=1.3, 95% CI 1.0-1.8) *(13)*.

Antibiotic Resistance Amongst Campylobacter spp.

Campylobacteriosis is a foodborne infection that is treated with fluids and oral antibiotics. Antibiotics are either bactericidal, those that kill the bacteria, or bacteriostatic, those that inhibit growth and allow for immune response for bacterial death. Additionally, antibiotics can be classified based upon their mechanism of action: inhibiting cell wall synthesis, protein synthesis, DNA synthesis, RNA synthesis, folate synthesis, or a combination. Penicillin and cephalosporin classes inhibit bacterial cell wall synthesis by inhibiting transpeptidase. Macrolides and tetracyclines are bacteriostatic, while aminoglycosides are bactericidal; however, these three classes all inhibit protein synthesis. Fluoro/quinolones block DNA synthesis by binding DNA gyrase *(38)*. Campylobacteriosis is commonly treated using erythromycin (a macrolide), tetracycline, or ciprofloxacin (a fluoro/quinolone) *(28)*.

Since the development of the National Antimicrobial Resistance Monitoring System-Enteric Bacteria (NARMS) in 1996, the presence of antibiotic resistance within human isolates, retail meats, and animals slaughtered for food has been monitored *(8)*. In 1999, Minnesota researchers investigated if there was a change in fluoro/quinolone- or nalidixic acid-resistance within Minnesota isolates from 1992 to 1998. Researchers found, among state isolates, resistance increased from 1.3% to 10.2% with the majority

of isolates being associated to international travel or taking preventative antibiotics while traveling or upon return *(28)*.

Notable Antibiotic Resistant Outbreaks

In recent years, there were two noteworthy *Campylobacter* outbreaks that revealed resistance to multiple antibiotics. Beginning in early August to October 2016, Colorado health officials identified a raw milk outbreak through a herd share program. Following interviews, twelve confirmed cases were found, ranging from 12-68 years in age, and resulted in one hospitalization and zero deaths. Cases ranged in age from 12-68 years. Case isolates (n=10) and milk samples (n=4) were analyzed using pulse-field gel electrophoresis (PFGE) for *C. jejuni*. All case isolates and two milk isolates were confirmed for *C. jejuni* and expressed resistance to ciprofloxacin, tetracycline, and nalidixic acid, following NARMS testing *(3)*.

In 2017, a *Campylobacter* outbreak related to puppies at pet stores involved 18 states and 118 cases. Based on case interviews, 105 cases reported a dog exposure with 95% reporting contact with a puppy at a pet store and eight reporting contact or purchasing a puppy from another pet store. This outbreak had 26 hospitalized cases and no deaths. Following a review of puppy records, puppies received prophylactic treatment (n=78), prophylactic and antibacterial treatment (n=54), or only antibacterial treatment (n=2). Of those antibiotics dispensed, broad-spectrum antibiotics, like doxycycline and azithromycin, accounted for 81%, in addition to sulfadimethoxine, a veterinary antibiotic. This brought attention to the use of antibiotic treatment within the commercial dog industry. Eighteen isolates were resistant to azithromycin, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, telithromycin, and tetracycline with some samples resistant to gentamicin and florfenicol *(18)*. Some of these antibiotics represent

the main treatment lines for campylobacteriosis patients, rendering these treatments as ineffective *(7)*.

For this current study, researchers aimed to understand to what extent *Campylobacter* spp. are present within the East Tennessee waterways, foodstuffs and in fecal material of wild and domestic animals within this region. *Campylobacter* isolates were speciated for *C. coli, jejuni* and *lari* to determine prevalence of the most commonly associated species for campylobacteriosis in addition to antibiotic resistance.

Materials and Methods

Microbiological Media

Four types of media were utilized to aid in the identification, enumeration, and isolation of *Campylobacter* spp. from locally grown foods, fecal samples, and water samples collected throughout East Tennessee.

Enumeration Broth

Bolton Broth (BB) (Oxoid, Bashingstoke, Hampshire, England) was used for the enumeration of *Campylobacter* spp. from locally growth foods and fecal samples collected throughout East Tennessee. It was prepared by following the company's instructions and was stored in sterilized glass bottles at refrigeration temperature until use. Upon use, broth bottles were brought to room temperature and enriched with Bolton Broth selective supplement (Oxoid, Bashingstoke, Hampshire, England) following manufacturer's instructions.

Enumeration Media

CHROMagar™ Campylobacter (CHROMagar™, Paris, France) was used for the selection and differentiation of *Campylobacter* species, by the presence of a red, pin-tip

colony on agar plates. CHROMagar™ Campylobacter allowed for the growth of *Campylobacter jejuni, Campylobacter coli,* and *Campylobacter lari,* which are most commonly linked to human illness.

Isolation Media

Mueller-Hinton (Oxoid, Basingstoke, Hamshire, England) was used as the media base for Mueller-Hinton Campylobacter blood plates for isolation. Manufacturer's instructions were followed. Once cooled, the following antibiotic supplements were added the media: (1) 16.0 ppm cefoperzone (Tokyo Chemical Industry Co, Portland, OR); (2) 1.0 ppm trimethoprim (Tokyo Chemical Industry Co, Portland, OR); (3) 1.0 ppm vancomycin hydrochloride (Alfa Aesar, Ward Hill, MA); (4) 0.5 ppm cycloheximide (VWR Life Science, Solon, OH); (5) 10% (v/v) defibrinated sheep's blood (HemoStat Laboratories, Dixon, CA).

Freezer Stock

Bolton broth (Oxoid, Basingstoke, Hampshire, England) made following manufacturer's instructions and 50% glycerol (Fisher Scientific, Fair Lawn, New Jersey) was used to create a 1.5 mL freezer stock solution. Samples with characteristic *Campylobacter* spp. growth were frozen for future use.

Sample Collection

Samples were obtained from eighteen counties within the greater East Tennessee geographical region.

Food Stuffs

Fresh dairy, meats, and produce were purchased from an area farmers' market monthly. These samples included: cheese, eggs, carrots, cucurbits, microgreens, radishes, sprouts, tomatoes, tree fruits, mushrooms, poultry, and beef. Samples were

purchased during bi-weekly farmers' market visits from June 2017 to August 2017 when available and transported back to the research facility for additional processing.

Fecal Samples

Samples of cattle, geese, and swine were collected from local parks and livestock processing facilities. At each site (n=7), a 36oz Whirl-Pak bag (Nasco, Fort Atkinson, WI) was filled halfway with feces. Bags were secured for aseptic transport back to the laboratory for further processing. Three samples were collected from each site monthly from March 2017 through July 2017.

Water Samples

At water sampling sites (Figure 2.1), a 36oz Whirl-Pak bag (Nasco, Fort Atkinson, WI) was connected to a 25-ft sampling line (Nasco, Fort Atkinson, WI) and thrown out into the water, skimming the surface as it was pulled back making sure to fill the bag three-fourths full.

From each collected sample, the temperature was recorded, at the time of collection, using a calibrated Traceable Waterproof thermometer (Fisher Scientific, Pittsburgh, PA), followed by fastening the sample for aseptic transport to the laboratory site. Three samples were collected at each site during each sampling day. Monthly water sampling occurred for both sites from March 2017 to July 2017.

Sample Processing

Food and Fecal Sample Isolation

A 25 g food or fecal sample was placed into a 36 oz Whirl-Pak bag (Nasco, Fort Atkinson, WI) along with 225 mL BB with supplements, made following manufacturer's instructions. Samples were incubated for 4 h at 37°C in a HERAcell 150i tri-gas incubator (ThermoFisher, Pittsburg, PA) with 5% O_2, 10% CO_2, and 85% N_2 (Airgas,

Knoxville, TN), then increased to 42°C for the remaining 44 h. Following incubation, using a 10 µL disposable inoculating loop/needle (ThermoFisher Scientific, Foster City, CA), samples were streaked for isolation onto CHROMagar™ Campylobacter base plates. Plates were inverted and incubated for 4 h at 37°C then 44 h at 42°C.

Water Sample Isolation

Using sterile forceps, a 47 mm, 0.45 µm sterile filter (PALL Life Sciences, Ann Arbor, MI) was placed onto a funnel base before volumes (10 mL or 100 mL) were pipetted using a 10 mL serological, sterile pipet (VWR, Atlanta, GA) or 100 mL serological, sterile pipet for filtration (CELLTREAT, Pepperell, MA), in duplicate. Following filtration, the filter was placed grid side up on CHROMagar™ Campylobacter base plates. Once all samples were processed, inverted plates were incubated as previously described. Plates with characteristic *Campylobacter* spp. growth according to CHROMagar™ interpretation guide (red, pin-tip colony) were categorized as presumptive positive.

Sample Confirmation

Isolation of Presumptive Campylobacter spp.

One presumptive *Campylobacter* colony was removed from CHROMagar™ Campylobacter base plates using a 10 µL disposable inoculating loop/needle (Thermo Fisher Scientific, Foster City, CA) and streaked for isolation onto Mueller-Hinton blood plates and incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2.

DNA Extraction using Boiling Lysis

To prepare for DNA extraction, an isolated colony from Mueller-Hinton blood plates was removed using a 10 µL disposable inoculating loop/needle (Thermo Fisher Scientific, Foster City, CA) and transferred into 10 mL Buffered Peptone Water (BPW;

BD Difco, Sparks, MD) and incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. Turbid tubes were removed, and 1.5 mL was transferred into an Eppendorf microcentrifuge tube (Eppendorf, Hauppauge, NY). Tubes were centrifuged at 10,000 x *g* for 5 minutes at 25°C using an AccuSpin Micro 17R microcentrifuge (Fisher Scientific, USA). Following centrifugation, the supernatant was discarded and 500 µL, 0.85% saline solution (Quality Biological, Gaithersburg, MD) was used to re-suspend the cell pellet then centrifuged at 10,000 x g for 3 minutes at 25°C. Again, the supernatant was discarded, and the pellet was re-suspended in 90 µL 1X Tris-EDTA (TE) buffer (Fisher, Fair Lawn, New Jersey). Cells were heated in a 97±2°C water bath for 15±1 minutes. Following heating, tubes were placed into refrigeration for 5 minutes then centrifuged a final time at 16,000 x g for 4 minutes at 25°C. Following centrifugation, without disturbing the pellet, the supernatant was transferred into a new, sterile microcentrifuge tube (Fisherbrand, Fair Lawn, New Jersey) and stored at -20°C until PCR analysis.

PCR

Four primer sets were used for PCR conformation to identify *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari* (Table 1), as described by Saiyudthong et al, 2015 *(26)*. For each 20 µL singleplex PCR reaction, the following was added to a sterile, Eppendorf microcentrifuge tube: 1.0 µM forward and reverse primers, 1 µL extracted DNA (with final concentrations ranging from 91-1265 ng/µL), 1X AmpliTaq Gold Fast PCR Mastermix (Applied Biosystems by ThermoFisher Scientific; Foster City, CA), and the remainder in nuclease free water (Fisher, Fair Lawn, NJ).

PCR analysis which was conducted in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Foster City, CA) with the following conditions: 35 cycles of 96˚C for 3 seconds, 62˚C for 3 seconds, 68˚C for 5 seconds; and a final elongation at 72˚C for 10

minutes. Samples were held at 4˚C until amplified products were visualized using gel electrophoresis.

Gel Electrophoresis

PCR products were examined for the presence of target sequences using an E-Gel EX 1% Agarose gel (Thermo Fisher Scientific, Foster City, CA) and read by the Invitrogen gel reader (Thermo Fisher Scientific, Foster City, CA) using a 1 Kb DNA ladder for reference (Thermo Fisher Scientific, Foster City, CA) and 10 µL 1X E-Gel Sample Loading Buffer (Thermo Fisher Scientific, Foster City, CA). Electrophoresis was conducted for 10 min at 48 V, 90 W and examined for characteristic bands at 1062 bp (16S rDNA), 773 bp (random sequence), 502 bp (*Aspartokinase* gene), or 251 bp (*glyA* gene) for *Campylobacter* spp., *C. jejuni, C. coli,* and *C. lari* identification, respectively *(26)*.

Presence of Campylobacter spp.

Following gel electrophoresis, samples that were confirmed as positive for the presence of *Campylobacter* spp. or one of the species-specific targets were identified. These samples were further analyzed to determine antibiotic resistance and to better understand the prevalence of antibiotic resistance within regional animals, food, and water.

Antimicrobial Testing of Confirmed Campylobacter spp.

Frozen sample cultures were streaked onto 5% TSA (BD Difco, Sparks, MD) with sheep's blood (HemoStat Laboratories, Dixon, CA) for isolation and incubated at 36°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. Following incubation, plates were swabbed using a sterile cotton swab (Puritan, Guilford, ME) and placed into 5mL Sensititre cation adjusted Mueller-Hinton broth tubes with TES buffer (CAMHBT) (Thermo Scientific,

Lenexa, KS). Each tubes was vortexed well and 100 µL were transferred into Sensititre CAMHBT + lysed horse blood (LBH) (Thermo Scientific, Lenexa, KS). Once vortexed, the tube was emptied into a sterile well basin and 100 µL were pipetted into each well of the CAMPY2 Sensititre plate (Thermo Scientific, West Sussex, UK). Plates were covered with a perforated adhesive seal, avoiding creases and making sure to place perforation over the well. Plates were incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. All plates were incubated within 30 minutes of starting the experiment and staked three high to maintain desired atmosphere and temperature. Plates were interpreted following incubation for turbidity or aggregation of cells at the bottom of the well.

Results and Discussion

Collection and Detection of Campylobacter spp.

During this preliminary study, samples (n=258) were collected from farmers' markets, livestock processing facilities, public parks, and surface river water (Figure 2.2). Results indicated presumptive presence of *Campylobacter* spp. in 47 (18%) samples and confirmed *Campylobacter* spp. presence in 43 (91%) of those samples. *C. coli* was isolated in twelve samples (28%) and *C. jejuni* in two (5%). *C. lari* was not isolated. The remaining 67% of isolates did not belong to one of these three species.

Over the five months, *Campylobacter* were isolated from surface river water (n=14, 67%), microgreens (n=5, 56%), swine feces (n=13, 54%), cattle feces (n=5, 21%), geese feces (n=4, 12%), sprouts (n=2, 8%). *Campylobacter* spp. were not isolated from tomatoes (n=24), cucurbits (n=21), beef cuts (n=18), poultry cuts (n=15),

eggs (n=12), tree fruits (n=12), raw cheese (n=6), raw milk (n=6), carrots (n=3), radishes (n=3), or mushrooms (n=3).

Campylobacter are traditionally linked to the consumption of raw and undercooked poultry and raw milk; however, this study shows that *Campylobacter* spp. isolation can be challenging and there might be additional environmental sources which may act as vehicles for human disease. After studying Canadian watersheds for three years, Van Dyke et al *(37)* detected *C. lari* in seagulls, ducks, and geese (32%) following PCR explaining the presence of *Campylobacter* within watersheds. Similarly, researchers at North Carolina State University identified *C. jejuni* (n=26) among urban Canadian geese samples (n=218) collected over a two-year period, of which all isolates were susceptible to six tested antibiotics *(25)*. This brings attention to a need at the regional or community-level for environmental assessments for additional campylobacteriosis risks.

In addition to animals for food and waterfowl being found to carry *Campylobacter* spp., this current study identified fresh produce also carrying *Campylobacter* spp. As previously mentioned, less than 1% of produce overall was estimated to be contaminated with bean and sprouts having the highest reported prevalence (11.08%) among fresh produce *(17)*. In this current study, microgreens (56%) and sprouts (8%) were found to be contaminated, while other produce, including tomatoes, cucurbits, tree fruits, carrots, radishes, and mushrooms showed no detectable growth of *Campylobacter* spp. These data were unlike the research by Doyle and Schoeni *(10)*.

Antibiotic Resistance

PCR confirmed isolates (n=39) were tested for antibiotic resistance according the CLSI guidelines adhering to the NARMS categorization of antibiotic resistance within

the 2010 NARMS Executive Summary (Table 2.3 and Table 2.4) *(35)*. Twenty isolates (51%) did not exhibit any growth at the lowest concentrations of all antibiotics evaluated. Of the remaining 19 isolates, all showed susceptibility to at least one antibiotic evaluated (Tables 2.3 and 2.4) *(24).*

Eleven isolates (28%) expressed antibiotic resistance, which is defined as an inability to successfully inhibit bacterial growth *in vitro* following a proper therapeutic dosage (Tables 3 and 4) *(24)*. Of these isolates, four (36%) were resistant to one antibiotic. These isolates were obtained from microgreens, river water from Site E, and swine feces. Three isolates (27%) were resistant to two antibiotics and were obtained from water at Sites C and E and cattle feces. Four isolates obtained from geese and swine feces were resistant to three or more antibiotics. One swine fecal isolate (9%) was resistant to three antibiotics. Two goose fecal samples showed resistance to five and six antibiotics, respectively, and a swine fecal sample isolate was resistant to seven antibiotics. No isolate expressed resistance to all eight antibiotics tested.

Amongst isolates obtained in this study, the greatest resistance was seen in nalidixic acid, a quinolone, with 18% of all isolates showing resistance (Table 2.4). Clindamycin and tetracycline resistance were observed in 15% of isolates. Macrolide antibiotics are most commonly prescribed for treating campylobacteriosis *(6)*. Three and eight percent of isolates obtained in this study were resistant to azithromycin and erythromycin, respectively, and were isolated from geese and swine feces (Table 2.4). Erythromycin resistance reported amongst *C. coli* and *jejuni* isolates analyzed through the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS), has remained below 4% for human and poultry isolates, but 13% of swine fecal samples

were exhibited resistance in 2015 *(36)*. Ciprofloxacin can also be used to treat campylobacteriosis *(6)*. Ciprofloxacin resistance from NARMS isolates have ranged from 0% in sows and turkeys to 10.4% in chicken and up to 50% in chicken *(36)*. Only 5% of isolates in this study were resistant to ciprofloxacin and gentamicin, respectively. Only one isolate obtained from geese feces expressed resistance to all three antibiotics commonly prescribed for treatment.

Other studies have found the highest resistance to azithromycin amongst isolates obtained from children and poultry (n=147) *(31)*. Conversely, only one isolate was resistant to this antimicrobial at therapeutic levels in this study (Table 2.3 and 2.4). Based upon these data, main treatment lines (erythromycin and ciprofloxacin) remain an option for more severe campylobacteriosis cases, which the CDC states resistance to azithromycin would be a serious public health threat *(5)*.

Fluoro/quinolone resistance was detected in a 2016 outbreak related to raw milk consumption in Colorado. Five of the ten confirmed case isolates were confirmed for resistance to ciprofloxacin, tetracycline, and nalidixic acid with two of these being a main treatment line for campylobacteriosis *(3)*. As previously mentioned, the 2016-2018 puppy store outbreak contained eighteen isolates susceptible to seven antibiotics: azithromycin, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, telithromycin, and tetracycline *(18)*. Bolinger and Kathariou revealed macrolide (azithromycin and erythromycin) resistance among *C. coli* isolates; this trend was only seen among one *C. coli* isolate obtained from swine feces which was resistant to erythromycin *(2)*. Additionally, researchers found pond isolates (85%) to be resistant to ciprofloxacin

unlike this preliminary study, which no river water (n=14) expressed resistance to ciprofloxacin *(31)*.

While the current study did not evaluate human isolates, there are *Campylobacter* species known to harbor the human oral cavity, such as *C. concisus, C. carvus, C. showae, C. rectus, and C. gracilis (20, 32)* which has led to discussions on non-*C. jejuni* infections. Campylobacteriosis is a leading cause of pathogenic diarrhea among children under 2 years of age and can lead to weight loss and hindered height growth among children 2 to 5 years old *(14, 15, 22, 23, 27)*. In a cohort of children, Schiaffino et al, *(27)* speciated 917 cultures of which 596 (65%) were *C. jejuni* isolates and 321 (35%) were non-*C. jejuni*. Of the non-*C. jejuni* isolates, 15.8% were resistant to gentamycin, while this current study identified 5% *(27)*. Additionally, multi-drug resistant (MDR) isolates accounted for 59.1% and this current study identified 54% (n=6) MDR isolates *(27)*.

As campylobacteriosis continues to be a leading bacterial foodborne illness, it is imperative that researchers have a better understanding of the number of sporadic cases. This study demonstrates the environmental reservoirs and the foodstuffs from which *Campylobacter* spp. can be isolated and exposures may occur at parks, rivers, and in locally grown food commodities. With the sporadic nature of campylobacteriosis, it is hard to understand what role non-*Campylobacter jejuni* and *coli* isolates may play with respect to human illness. Even self-limiting diarrheal illnesses negatively impact productivity. Isolates obtained in this study should be further characterized to better understand what pathogenicity elements they have in addition to the currently characterized antimicrobial resistance. Additionally, this study emphasizes the

importance of antimicrobial stewardship as campylobacteriosis continues to be a leading bacterial foodborne illness. *Campylobacter* spp. have shown capability of developing antibiotic resistance. The loss of effective antibiotic treatment would be detrimental to public health.

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Chapter III: Enumeration, Isolation, and Detection of *Campylobacter* species within the Tennessee River throughout the Greater Knoxville Area

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Abstract

Campylobacter spp. are one of the main causes of bacterial gastroenteritis in the United States. Campylobacteriosis is commonly associated with the consumption of poultry, meat, and raw milk; however, following a preliminary study, researchers quantified populations of *Campylobacter* spp. within the Tennessee River near Knoxville, TN. This study aimed to better understand the spatiotemporal distribution of *Campylobacter* spp. and further elucidate surface water as a possible vehicle for campylobacteriosis by sampling four sites (A-D) of the Tennessee River. Samples (n=264) were collected from four public access river sites for one calendar year (November 2017-November 2018). Samples were vacuum filtered through a 0.45µm membrane filter and plated onto Campy CHROMagar®. Plates were incubated in a microaerophilic environment (5% O_2, 10% CO_2, 85% N_2) for 4 h at 37˚C then 44 h at 42˚C for isolation of *Campylobacter*. Presumptive positives (n=244) were confirmed via PCR with targets for *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari*. Confirmed isolates were identified as *Campylobacter* spp. (n=168) and *C. coli* (n=5). All collection sites had a relatively high of *Campylobacter* spp. isolated [site A (n=30/38; 79%), site B (n=29/38; 76%), site C (n=77/132; 58%), and site D (n=32/36; 89%)]. Five water samples were confirmed as *C. coli* from site D, which were obtained from January to March. PCR confirmed samples (n=161) were tested for antibiotic resistance amongst a panel of eight antibiotics. Ninety-five (59%) isolates expressed resistance, of which 23 (14%) isolates were resistant to one antibiotic, 27 (17%) isolates were resistant to two, 18 (11%) were resistant to three, 12 (7%) were resistant to four, three (2%) were resistant to five, four (2%) were resistant to six, and five (3%) were resistant to seven,

and three (2%) were resistant to all eight. These data indicate that antibiotic resistant *Campylobacter* spp. are present within the surface waters of the Tennessee River throughout the Knoxville area. This study shows a need to update campylobacteriosis risk factors, campylobacteriosis prevention education, and future treatment options.

Introduction

Overview

Campylobacter species (*Campylobacter* spp.) are microaerophilic, Gram-negative, curved rods with a single, polar flagellum *(13, 38)*. Originally recognized as the cause of veterinary spontaneous abortions, *Campylobacter* spp. are commensals within the intestinal track of warm-blooded animals *(2, 38)*. In 1978, Bennington, Vermont experienced the first known *Campylobacter* outbreak related to the contamination of the city's municipal water supply *(41)*. Campylobacteriosis is a gastrointestinal infection occurring 24-48 hours after consumption. Symptoms include abdominal pain, cramps, diarrhea, malaise, headaches, or a fever. These symptoms usually abate 2-7 days, depending on one's immune state, age, and number of *Campylobacter* consumed. The microorganism can be shed for months following the cessation of symptoms *(13)*. Campylobacteriosis can lead to post-infection sequelae, including Guillain-Barré syndrome (GBS), reactive arthritis (ReA) or Reiter's Syndrome, and irritable bowel syndrome (IBS). FoodNet reported in 2016 that *Campylobacter* had the highest incidence of foodborne infections with 11.79 confirmed cases per 100,000 persons; 99% of cases occur sporadically with peaks from May-August *(18, 40)*.

Reservoirs

First classified as *Vibrio*, now there are 27 known species of *Campylobacter* and eight subspecies, with *Campylobacter jejuni (C. jejuni)* causing the greatest number of human campylobacteriosis cases *(13, 17, 22)*. The Centers for Disease Control and Prevention (CDC) began active surveillance for *Campylobacter* spp. in 1982, and since campylobacteriosis outbreaks have been linked to water and food *(10, 11, 24, 35, 36, 38, 40)*. Currently, the CDC recognizes raw or undercooked poultry, cross

contamination with raw or undercooked poultry, raw dairy products, untreated water, and produce as risk for campylobacteriosis *(6)*. Given that *Campylobacter* spp. are commensals within warm-blooded animals, colonization of the gut can occur in birds within 2-3 weeks following hatching, and an infection can spread throughout an entire flock in 3 days *(21)*. Poultry flock surveys report that 95% of flocks are positive for *Campylobacter* spp. *(21)*.

Campylobacter and Water

As mentioned earlier, the first known *Campylobacter* outbreak in the United States occurred in Bennington, VT in 1978 due to a system breach and a lack of free chlorine levels within a city's municipal water supply *(41)*. In 1983, staff and students (n=257) at a boarding school near Essex, United Kingdom experienced signs of gastroenteritis. Survey results identified an association with consumption of water from the school among staff (54%, $p < 0.01$) and students (11%, $p < 0.01$). Isolates (n=10) were sent for additional testing where six samples identified to have a common serotype and two strains were similar to those found in collected water samples from the school water supply *(23)*. Similar to these outbreaks, researchers noticed high levels of gastroenteritis among wilderness hikers in the Grand Teton National Park in Jenny Lake, Wyoming during the summers of 1980 and 1981. *C. jejuni* was identified in 23% of hikers with gastroenteritis one week following the consumption of untreated surface water *(39)*. To date, the largest United States waterborne *Campylobacter* outbreak occurred in 1999 in Washington County, New York. This outbreak resulted in over 116 *Escherichia coli* O157:H7 infections and 13 co-infections with *C. jejuni (5)*. Following a case-control study, public health officials identified 26/32 (81%) cases and 9/57 (16%) controls consumed water during the days in question; therefore, officials concluded that

“drinking water or beverages made with water from the suspect well was associated with illness” *(5)*. Similar outbreaks have been experienced in Canada, New Zealand, and Scotland.

In 1985, southern Ontario experienced an outbreak of 241 gastroenteritis cases from March 29-April 17. This resulted in 57 confirmed *C. jejuni* cases, and the outbreak investigation did not reveal any age or gender specific patterns. Following a case-control study, those who consumed more glasses of water per day were more likely to have become ill (avg 4.6 glasses for cases vs avg 3.4 glasses for control) *(19)*. While the epidemic curve displayed a common source outbreak, routine well water testing identified pathogenic contamination even though contamination was not expected, since this outbreak occurred in the springtime as ground snow was melting and few farm animals from wading. This outbreak brings light to *Campylobacter* spp. ability to survive in low ambient temperatures *(19)*. A second waterborne Canadian outbreak occurred in 2000 in Walkerton, Ontario. It was hypothesized that the Walkerton municipal water supply was contaminated via area farms following heavy rains, which allowed manure to enter the groundwater supply. Following testing of area farms, isolates expressed similar *C. jejuni* and *C. coli* strains to those linked to outbreak isolates *(9)*.

The first known New Zealand waterborne *Campylobacter* outbreak occurred in 1990 outside Christchruch at a camp and conference center where two reports of *C. jejuni* were identified *(37)*. Following interviews (n=99) of all camp leaders, staff, family, and campers, 44 cases were identified with 11 cases (25%) matching the case definition, 14 (32%) due to diarrhea history, and 19 (43%) due to expression of four or more symptoms *(37)*. A case-control study was conducted which identified consumption

of ≥ 2 cups of non-boiled water a day *(37)*. A second New Zealand outbreak occurred in Darfield, a rural community, in 2012. This outbreak resulted in 29 cases due to contaminated ground water supply due to nearby animal feces following heavy rains. While New Zealand had water regulations and standards at the time of this outbreak, it was not required for all water supplies, such as the one in Darfield *(1)*. A third and largest documented outbreak occurred in Hastings, New Zealand in 2016 when one-third of 14,000 residents were diagnosed with campylobacteriosis, in which three individuals died, and one person was diagnosed with Guillain-Barré *(27, 28)*. This outbreak was recognized at the same time a positive groundwater test was identified. The New Zealand government implemented chlorination of groundwater supplies and put a water boil advisory into effect *(27)*. It is believed that sheep feces was likely the cause of contamination *(28)*.

As a final example, a waterborne outbreak of *Campylobacter* occurred in 1995 in Fife, Scotland when 711 of the 1100 residents reported gastroenteritis resulting in 633 cases *(14)*. Of the cases, 70 stool samples were tested and resulted in the following identification – *Campylobacter* spp. (n=9), Rotavirus (n=1), and *Escherichia coli* O157 (n=6) *(14)*. While the village followed Scotland's Water Supply Regulations of 1990, backflow from a local produce processor had contaminated the village's water *(14)*.

These outbreaks demonstrate the ability of water to act as a reservoir for *Campylobacter* spp., especially when influenced by runoff from animal feces. Furthermore, these outbreaks elucidate the importance of water standards and multi-barrier approaches, as well as routine testing of potable water for the prevention of pathogenic waterborne outbreaks.

Antibiotic Overview

Campylobacteriosis is commonly treated with oral antibiotics and fluids. Antibiotics are bactericidal, those that kill bacteria, or bacteriostatic, those that inhibit growth and allow for immune response for bacterial death. Antibiotics are further classified based upon their mechanism of action: inhibition of cell wall synthesis, protein synthesis, DNA synthesis, RNA synthesis, folate synthesis, or a combination. Penicillin and cephalosporin classes inhibit bacterial cell wall synthesis by inhibiting transpeptidase. Macrolides and tetracyclines are bacteriostatic, while aminoglycosides are bactericidal; however, these three classes all inhibit protein synthesis. Fluoro/quinolones block DNA synthesis by binding DNA gyrase *(45)*. With over 20 classes of antibiotics, campylobacteriosis is commonly treated using erythromycin (a macrolide), tetracycline, or ciprofloxacin (a fluoro/quinolone) *(34)*.

The National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) was established in 1996 in order to monitor antibiotic resistance within human isolates, retail meats, and animals for food *(8)*. Since testing began in 1996, NARMS reports indicate erythromycin resistance is under 4% among human and chicken for *C. jejuni* isolates. This has remained true for *C. jejuni* isolates in the 2015 NARMS Integrated Report *(43)*. However, erythromycin resistance among human, chicken, and retail chicken *C. coli* isolates has increased since 2011 from 2.7% (humans), 3.4% (chicken), 5.2% (retail chicken) to 12.7% (humans), 12.8% (chicken), and 12.7% (retail chicken) *(43)*.

Notable Antibiotic Resistant Outbreaks

In recent years, there were two noteworthy *Campylobacter* outbreaks that revealed resistance to multiple antibiotics. Beginning in early August to October 2016,

Colorado health officials identified a raw milk *C. jejuni* outbreak through a herd share program. Following interviews, twelve confirmed cases were found, ranging from 12-68 years in age, and resulted in one hospitalization and zero deaths. Cases ranged in age from 12-68 years. Case isolates (n=10) and milk samples (n=2) were linked for *Sma* enzyme following pulse-field gel electrophoresis (PFGE) analysis for *C. jejuni*. All isolates that were screened expressed resistant to ciprofloxacin, tetracycline, and nalidixic acid *(4)*.

In 2017, a *Campylobacter* outbreak related to puppies at pet stores involved 118 cases across 18 states. One hundred five cases reported a dog exposure, with 95% reporting contact with a puppy at a pet store, and eight reporting contact or purchasing a puppy from another pet store. This outbreak had 26 hospitalized cases. Following a review of puppy records, puppies received prophylactic treatment (n=78), prophylactic and antibacterial treatment (n=54), or only antibacterial treatment (n=2) of at least one antibiotic. Of those antibiotics dispensed, broad-spectrum antibiotics, like doxycycline and azithromycin, accounted for 81%, in addition to sulfadimethoxine, a veterinary antibiotic. This brought attention to the use of antibiotic treatment within the commercial dog industry. Eighteen isolates were resistant to azithromycin, ciprofloxacin, clindamycin, erythromycin, nalidixic acid, telithromycin, and tetracycline with some samples resistant to gentamicin and florfenicol *(20)*. Azithromycin and ciprofloxacin are main treatment lines for campylobacteriosis patients *(7)*.

For this current study, researchers aimed to understand to what extent *Campylobacter* spp. are present within the Tennessee River within Blount and Knox counties in Tennessee. *Campylobacter* were speciated for *C. coli, jejuni,* and *lari* to

determine prevalence of the most commonly associated species for campylobacteriosis in addition to antibiotic resistance.

Materials and Methods

Microbiological Media

Three types of media were utilized to aid in the identification, enumeration, and isolation of *Campylobacter* spp. from water samples collected.

Enumeration Media

CHROMagar™ Campylobacter (CHROMagar™, Paris, France) was used for the selection and differentiation of *Campylobacter* species, by the presence of a red, pin-tip colony on agar plates. CHROMagar™ Campylobacter allowed for the growth of *Campylobacter jejuni, Campylobacter coli,* and *Campylobacter lari,* which are most commonly linked to human illness.

Isolation Media

Mueller-Hinton (Oxoid, Basingstoke, Hamshire, England) was used as the media base for Mueller-Hinton *Campylobacter* blood plates for isolation. Manufacturer's instructions were followed. Once cooled, the following antibiotic supplements were added the media: (1) 16.0 ppm cefoperzone (Tokyo Chemical Industry Co, Portland, OR); (2) 1.0 ppm trimethoprim (Tokyo Chemical Industry Co, Portland, OR); (3) 1.0 ppm vancomycin hydrochloride (Alfa Aesar, Ward Hill, MA); (4) 0.5 ppm cycloheximide (VWR Life Science, Solon, OH); (5) 10% (v/v) defibrinated sheep's blood (HemoStat Laboratories, Dixon, CA).

Freezer Stock

Muller-Hinton broth (Oxoid, Basingstoke, Hampshire, England) made following manufacturer's instructions and 50% glycerol (Fisher Scientific, Fair Lawn, New Jersey)

was used to create a 1.5 mL freezer stock solution. Samples with characteristic *Campylobacter* spp. growth were frozen for future use.

Sample Collection

Water Samples

At each sampling site [A, B, C, and D (Figure 3.1)], a 36oz Whirl-Pak bag (Nasco, Fort Atkinson, WI) was connected to a 25-ft sampling line (Nasco, Fort Atkinson, WI) and thrown out into the water, skimming the surface as it was pulled back making sure to fill the bag three-fourths full.

From each collected sample, the temperature was recorded, at the time of collection, using a calibrated Traceable Waterproof thermometer (Fisher Scientific, Pittsburgh, PA), followed by fastening the sample for aseptic transport to the laboratory. Three samples were collected at each site during each sampling day. Targeted weekly sampling occurred for Site C from November 2017 to November 2018, encompassing an entire year. Beginning in late-July 2018, sites A, B, and D were added to weekly collection until the end of November 2018.

Sample Processing

Direction Enumeration

Using sterile forceps, a 47 mm, 0.45 µm sterile filter (PALL Life Sciences, Ann Arbor, MI) was placed onto a funnel base before volumes (10 mL or 100 mL) were pipetted using a 10 mL serological, sterile pipet (VWR, Atlanta, GA) or 100 mL serological, sterile pipet for filtration (CELLTREAT, Pepperell, MA), in duplicate. Following filtration, the filter was placed grid side up on CHROMagar™ Campylobacter base plates. Once all samples were processed, inverted plates were incubated for 4 h at 37°C in a HERAcell 150i tri-gas incubator (ThermoFisher, Pittsburg, PA) with 5% O_2,

10% CO_2, and 85% N_2 (Airgas, Knoxville, TN), then increased to 42°C for the remaining 44 h. Plates with characteristic *Campylobacter* spp. growth according to CHROMagar™ interpretation guide (red, pin-tip colony) were enumerated to indicate the number of presumptive *Campylobacter* spp. present for each collection site. For any sample, which at the lowest volume of water (10 mL or 100 mL) had greater than 250 colonies, but had 5-10 colonies per square, 10 random squares were counted, added together, divided by 10, then multiplied by 80 (filter area) to estimate *Campylobacter* population.

Sample Confirmation

Isolation of Presumptive Campylobacter spp.

A presumptive *Campylobacter* colony was removed from CHROMagar™ Campylobacter base plates using a 10 µL disposable inoculating loop/needle (Thermo Fisher Scientific, Foster City, CA) and streaked for isolation onto Mueller-Hinton blood plates and incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2.

DNA Extraction using Boiling Lysis

To prepare for DNA extraction, an isolated colony from Mueller-Hinton blood plates was removed using a 10 µL disposable inoculating loop/needle (Thermo Fisher Scientific, Foster City, CA) and transferred into 10 mL Buffered Peptone Water (BPW; BD Difco, Sparks, MD) and incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. Turbid tubes were removed, and 1.5 mL was transferred into an Eppendorf microcentrifuge tube (Eppendorf, Hauppauge, NY). Tubes were centrifuged at 10,000 x *g* for 5 minutes at 25°C using an AccuSpin Micro 17R microcentrifuge (Fisher Scientific, USA). Following centrifugation, the supernatant was discarded and 500 µL, 0.85% saline solution (Quality Biological, Gaithersburg, MD) was used to re-suspend the cell pellet then centrifuged at 10,000 *x g* for 3 minutes at 25°C. Again, the supernatant was

discarded, and the pellet was re-suspended in 90 µL 1X Tris-EDTA (TE) buffer (Fisher, Fair Lawn, New Jersey). Cells were heated in a 97±2°C water bath for 15±1 minutes. Following heating, tubes were placed into refrigeration for 5 minutes then centrifuged a final time at 16,000 x g for 4 minutes at 25°C. Following centrifugation, without disturbing the pellet, the supernatant was transferred into a new, sterile microcentrifuge tube (Fisherbrand, Fair Lawn, New Jersey) and stored at -20°C until PCR analysis.

PCR

As described by Saiyudthong et al, 2015, four primer sets were used for PCR conformation to identify *Campylobacter* spp., *C. jejuni*, *C. coli*, and *C. lari* (Table 3.1) *(31)*. For each 20 µL singleplex PCR reaction, the following was added to a sterile, Eppendorf microcentrifuge tube: 1.0 µM forward and reverse primers, 1 µL extracted DNA (with final concentrations ranging from 91-1265 ng/µL), 1X AmpliTaq Gold Fast PCR Mastermix (Applied Biosystems by ThermoFisher Scientific; Foster City, CA), and the remainder in nuclease free water (Fisher, Fair Lawn, NJ).

PCR analysis was conducted in a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Foster City, CA) using the following conditions: 35 cycles of 96˚C for 3 seconds, 62˚C for 3 seconds, 68˚C for 5 seconds; and a final elongation at 72˚C for 10 minutes. Samples were held at 4˚C until amplified products were visualized using gel electrophoresis.

Gel Electrophoresis

PCR products were examined for the presence of target sequences using an E-Gel EX 1% Agarose gel (Thermo Fisher Scientific, Foster City, CA) and read by the Invitrogen gel reader (Thermo Fisher Scientific, Foster City, CA) using a 1 Kb DNA ladder for reference (Thermo Fisher Scientific, Foster City, CA) and 10 µL 1X E-Gel

Sample Loading Buffer (Thermo Fisher Scientific, Foster City, CA). Electrophoresis was conducted for 10 min at 48 V, 90 W and examined for characteristic bands at 1062 bp (16S rDNA), 773 bp (random sequence), 502 bp (*Aspartokinase* gene), or 251 bp (*glyA* gene) for *Campylobacter* spp., *C. jejuni, C. coli,* and *C. lari* identification, respectively *(31)*.

Presence of Campylobacter spp.

Following gel electrophoresis, samples that were confirmed as positive for the presence of *Campylobacter* spp. or for one of the species-specific targets were identified. These samples were further analyzed to determine antibiotic resistance and to better understand the prevalence of antibiotic resistance on a temporal and spacial basis.

Antimicrobial Testing of Confirmed Campylobacter spp.

Frozen sample cultures were streaked onto 5% TSA (BD Difco, Sparks, MD) with sheep's blood (HemoStat Laboratories, Dixon, CA) for isolation and incubated at 36°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. Following incubation, plates were swabbed using a sterile cotton swab (Puritan, Guilford, ME) and placed into 5mL Sensititre cation adjusted Mueller-Hinton broth tubes with TES buffer (CAMHBT) (Thermo Scientific, Lenexa, KS). Each tube was vortexed well and 100 µL were transferred into Sensititre CAMHBT + lysed horse blood (LBH) (Thermo Scientific, Lenexa, KS). Once vortexed, the tube was emptied into a sterile well basin and 100 µL were pipetted into each well of the CAMPY2 Sensititre plate (Thermo Scientific, West Sussex, UK). Plates were covered with a perforated adhesive seal, avoiding creases and making sure to place perforation over the well. Plates were incubated at 42°C for 24 h with 5% O_2, 10% CO_2, and 85% N_2. All plates were incubated within 30 minutes of starting the experiment and

staked three high to maintain desired atmosphere and temperature. Plates were interpreted following incubation for turbidity or aggregation of cells at the bottom of the well.

Results and Discussion

Collection and Detection of Campylobacter spp.

Sample collection began in November 2017 for Site C resulting in 147 collected samples of which 75 were confirmed utilizing PCR as *Campylobacter* spp. (n=70) and *C. coli* (n=5). Sites A, B, and D began collection in July 2018 and resulted in 320 samples. Following PCR confirmation, Sites A, B, and D consisted of only *Campylobacter* spp. (n=30, 32, and 29 respectively). Data show that even as water temperatures drop throughout the winter, *Campylobacter* are still present (6.1°C-32.4°C). In SAS, a mixed models linear regression and post hoc for means separation was used to identify if a statistically significant difference ($p<0.05$) existed between months (November 2017 to November 2018). There is a statically significant difference ($p=0.0229$) among mean log counts/100 µL by month at site C (Figure 3.2). Additionally, when looking at a means separation among all collection sites (A, B, C, and D) for five months (July 2017 to November 2017). There is a statistically significant difference ($p=0.0021$) among mean log counts/100 µL for July 2017 to September 2017 as compared to the average counts from October 2017 and November 2017 (Figure 3.3). Research by Korhonen and Martikainen *(15)* found that *C. jejuni* survive better at 4°C than 25°C, could explain high levels of *Campylobacter* spp. during winter months. Additionally, the 1985 Ontario outbreak identified the capability of *Campylobacter* spp. to survive at lower temperatures than previously believed *(19)*. In Finland, Schönberg-

Norio et al *(33)* performed a matched case-control study (n=237) that identified three independent risk factors of campylobacteriosis: 1) tasting or eating raw or undercooked meat (OR 10.76, 95% CI 1.31-89.09); 2) drinking water from a dug well (OR 3.36, 95% CI 1.37-8.24); and 3) swimming in water from natural sources (OR 2.80, 95% CI 1.23-6.39) all at $p < 0.05$. This study helps to substantiate the exposure to river water as a cause of sporadic campylobacteriosis.

This study identified non-*C. jejuni* and non-*C. coli* isolates among the Tennessee River watershed (Table 3.3). Similarly, Finnish researchers identified *C. lari* and unidentified *Campylobacter* spp. among bathing and purified sewage water samples in addition to *C. coli* and *C. jejuni*. It was noted that *Campylobacter* spp. samples were significantly influenced by bathing water temperatures ($p < 0.05$) *(12)*. In addition to water, non-*C. jejuni* isolates have been identified among Peruvian children according to a study by Schiaffino et al *(32)*. In a children cohort, 917 cultures were speciated as *C. jejuni* (596, 65%) and non-*C. jejuni* (321, 35%). It is reported that campylobacteriosis is a leading cause of pathogenic diarrhea among children under 2 years of age leading to weight loss and in children 2 to 5 years old a decrease in height *(16, 17, 25, 26, 32)*.

Antibiotic Resistance

PCR confirmed samples (n=161) were tested for antibiotic resistance (Table 3.3). Fifty-three samples (33%) could not proliferate within the SENSITIRE CAMPY2 plates, indicating susceptibility to the lowest concentrations of antimicrobials evaluated, while 67% (n=108) expressed antibiotic susceptibility, intermediate growth, or resistance according to the CLSI guidelines within the 2010 NARMS Executive Summary (Table 3.4) *(42)*.

Ninety-five (59%) isolates expressed resistance or an inability to hinder bacterial growth *in vitro* to at least one antibiotic at therapeutic doses (Tables 3.3 and 3.4) *(29)*. According to the 2010 NARMS Executive Summary, erythromycin resistance has remained around 4%, ciprofloxacin resistance remained ≥20%, and gentamicin around 1%. In comparison to the NARMS Summary, the current study found highest resistance to clindamycin (47%) followed by nalidixic acid (43%) *(42)*. Twenty-three (14%) isolates were resistant to one antibiotic, 27 (17%) isolates were resistant to two, 18 (11%) were resistant to three, 12 (7%) were resistant to four, three (2%) were resistant to five, four (2%) were resistant to six, and five (3%) were resistant to seven (Figure 3.3). Three isolates, all collected on July 28, 2018, at sites B (2) and C, expressed resistance to all eight antibiotics. Four isolates [Site B (7/28/18), Site B (7/28/18), Site C (7/28/18), Site D (8/12/18)] were resistant to the three antibiotics (erythromycin, ciprofloxacin, and tetracycline), which are commonly prescribed for campylobacteriosis (Figure 3.4) *(6)*. These isolates indicate dissemination throughout the Tennessee River in Blount and Knox Counties, TN. These results are similar to research conducted by Rutledge et al and Van Dyke et al *(30, 44)*. Rutledge et al *(30)* which identified 26 *Campylobacter* positive isolates at seven sites throughout Guildford Co, North Carolina during a two-year window, attributed to the migration of Canadian geese. Van Dyke et al *(44)* identified 394 *Campylobacter* isolates within five collection sites throughout the Grand River watershed in Ontario, Canada over three years. While these studies were longer than the one-year time frame of our study, all studies indicate the likelihood of isolates traveling throughout watersheds.

Bolinger and Kathariou revealed macrolide (azithromycin and erythromycin) resistance to be common among *C. coli* isolates; however, of the nine isolates resistant to both macrolides, none of the isolates were speciated as *C. coli (3)*. Lastly, Schiaffino et al *(32)* studied 596 isolates of which 59.1% were found to be multi-drug resistant (MDR). In this current study, 73 (45%) isolates were identified as MDR.

Conclusion

This study has substantiated the survival of *Campylobacter* spp. in river surface water throughout the year, indicating that water can act as an important reservoir for disseminating *Campylobacter* spp. Additionally, this study shows multiple-antibiotic resistance amongst *Campylobacter* spp., which can both complicate medical treatment, if the organism is capable of causing infections, or serve as a reservoir for antibiotic-resistant genetic elements that can be disseminated to other microorganisms present within the environment. Further evaluation of the public health importance of non-*C. jejuni, coli,* and *lari Campylobacter* should be determined through whole genome sequencing and *in vivo* analysis to better characterize what virulence elements these organisms have and if they are able to cause diarrheal or other illnesses.

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Dissertation Conclusion

Campylobacter spp. remain a threat to public health and the food industry as a leading cause of bacterial foodborne illness. This study identified the presence of *Campylobacter* spp. within previously undocumented food stuffs, such as sprouts and microgreens, and in animal feces, such as swine. Additionally, this study provided a better understanding of the spatiotemporal distribution of *Campylobacter* spp. and the possibility of surface water serving as a vehicle for campylobacteriosis, especially sporadic cases with no epidemic link. Part one of this study indicated antibiotic resistant *Campylobacter* spp. present in surface waters, animal feces, and sprouted seeds within the greater East Tennessee geographical region. Part two of this study indicated the presence of antibiotic resistant *Campylobacter* spp. within the surface waters of the Tennessee River within Blount and Knox Counties. Both of these could result in negative implications for treatment and overall health complications for the region.

By completing this study, a need to update campylobacteriosis risk factors, prevention education, and future treatment options has been identified, especially among TDH surveillance areas.

Appendices

Appendix A

Table 1.1. Currently recognized *Campylobacter* species and associated hosts modified from Ngulukun *(33)*

Species and Subspecies	Host
C. fetus	
fetus	Cattle, sheep
venerealis	Cattle, sheep
C. jejuni	
jejuni	Poultry, cattle, humans
doylei	Humans
C. coli	Pigs
C. concicsus	Humans
C. carvus	Humans
C. hyopinestinalis	
hyoinestinalis	Pigs, cattle, humans
lawsonii	Pigs
C. lari	
lari	Seagulls, dogs, humans
concheus	Humans, shellfish
C. rectus	Humans
C. upsaliensis	Cats, dogs, monkeys
C. helveticus	Cats, dogs
C. gracilis	Humans
C. showae	Humans
C. sputorum	Cattle, pigs, humans
C. lanienae	Pigs
C. hominis	Humans
C. mucosalis	Pigs
C. insulaenigrae	Seals, porpoise
C. canadensis	Wild birds
C. cuniculorum	Rabbits
C. peloridis	Humans, mollusks
C. avium	Poultry
C. ureolyticus	Humans
C. volucris	Wild birds, humans
C. subantarcticus	Wild birds
C. troglodytis	Chimpanzees
C. corcagiensis	Captive lion-tailed macaques
C. iquaniorum	Lizards, chelonians

Appendix B

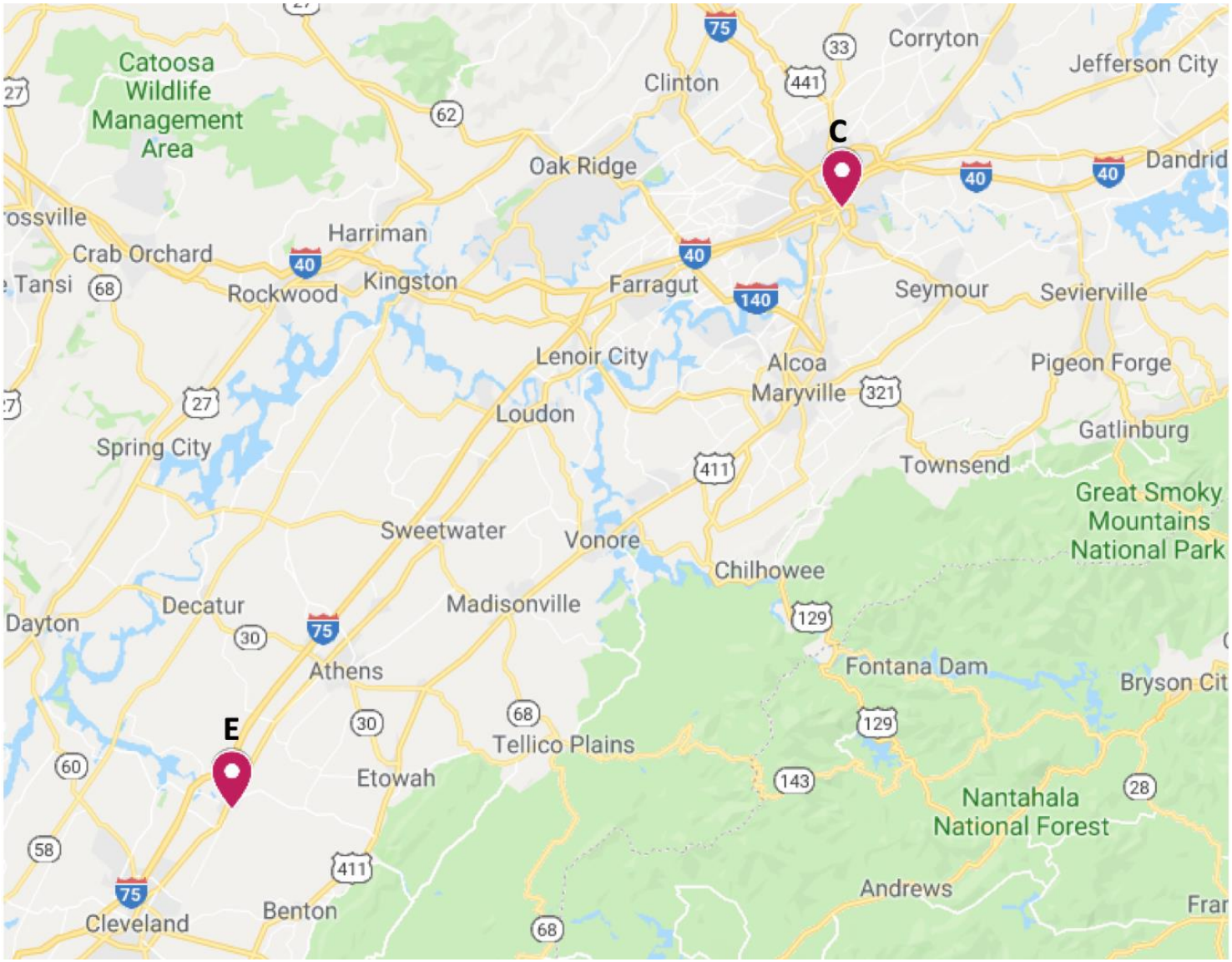

Figure 2.1 Hiwassee River Water Collection Site E and Tennessee Rivers Water Collection Sites

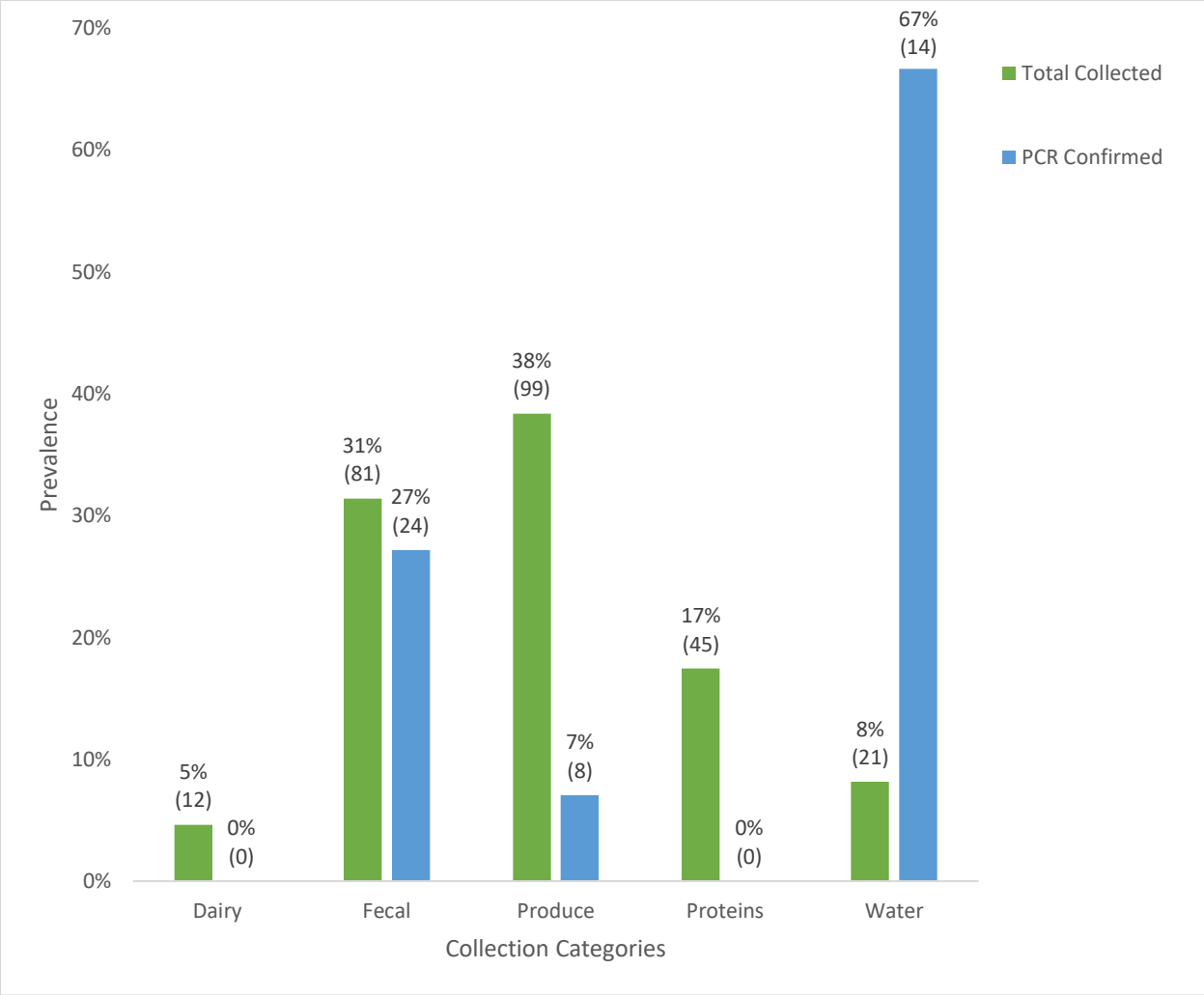

Figure 2.2. Prevalence of confirmed *Campylobacter* spp. among locally collected food, fecal, and water samples from March 2017-August 2017

Table 2.1. PCR primer sequences selected from Saiyudthong et al, 2015 *(26)*

	Primer	Sequence (5’ to 3’)	Size (bp)
Campylobacter	Campy-F	GGA GGC AGC AGT AGG GAA TA	1,062
	Campy-R	TGA CGG GCG GTG AGT ACA AG	
C. jejuni	Jejuni-F	CAT CTT CCC TAG TCA AGC CT	773
	Jejuni-R	AAG ATA TGG CTC TAG CAA GAC	
C. coli	Coli-F	GGT ATG ATT TCT ACA AAG CGA G	502
	Coli-R	ATA AAA GAC TAT CGT CGC GTG	
C. lari	Lari-F	TAG AGA GAT AGC AAA AGA GA	251
	Lari-R	TAC ACA TAA TAA TCC CAC CC	

Table 2.2. PCR reaction well components

AmpliTaq Gold Fast PCR, UP	10 µL
Forward Primer	2 µL
Reverse Primer	2 µL
dH_2O	5 µL
Sample extracted DNA	1 µL

Table 2.3. Minimal Inhibitory Concentrations (MICs) of *Campylobacter* spp. confirmed samples using Sensitire CAMPY2 plates

Isolate	Sample[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycosides	Lincosamides	Macrolides		Phenicols	Quinolones		Tetracyclines
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
C. coli	SF	ng[E]	4 I	0.5 S	2 S	1 S	0.5 S	**>64 R**	2 S
C. coli	SF	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	SF	ng	2 S	0.12 S	1 S	1 S	0.12 S	16 S	2 S
C. coli	SF	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	SF	**64 R**	**32 R**	2 S	**32 R**	**>64 R**	**64 R**	**>64 R**	**>64 R**
C. coli	SF	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	SF	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	SF	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	SF	ng	2 S	0.06 S	1 S	1 S	0.06 S	8 S	16 R
C. jejuni	CF	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	CF	ng	**32 R**	1 S	**16 I**	4 S	ng	**64 R**	1 S
Campy spp	CF	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	CF	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	CF	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	GF	ng	ng	ng	ng	ng	ng	ng	**ng**
Campy spp	GF	4 I	**32 R**	**>64 R**	**>64 R**	4 S	**32 R**	**>64 R**	**>64 R**
Campy spp	GF	**8 R**	**32 R**	2 S	**32 R**	**64 R**	ng	8 S	**64 R**
Campy spp	MG	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	MG	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	MG	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	MG	1 S	2 S	0.5 S	16 I	2 S	0.12 S	**64 R**	2 S
Campy spp	MG	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	SF	ng	**16 R**	1 S	8 S	**8 R**	ng	ng	**16 R**
Campy spp	Site C	ng	4 I	0.12 S	1 S	4 S	0.12 S	16 S	1 S
Campy spp	Site C	ng	ng	ng	ng	ng	ng	ng	ng

Table 2.3 Continued

Isolate	Sample[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycosides	Lincosamides	Macrolides		Phenicols	Quinolones		Tetracyclines
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	Site C	2 S	**32 R**	0.25 S	1 S	1 S	0.12 S	**64 R**	1 S
Campy spp	Site C	ng[E]	4 I	0.25 S	4 S	2 S	0.12 S	16 S	1 S
Campy spp	Site C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	Site C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	Site C	ng	2 S	0.12 S	1 S	1 S	0.5 S	16 S	1 S
Campy spp	Site C	ng	2 S	0.06 S	0.25 S	0.12 S	0.25 S	16 S	ng
Campy spp	Site E	1 S	4 I	0.25 S	4 S	4 S	0.5 S	32 I	2 S
Campy spp	Site E	ng	4 I	0.25 S	4 S	**8 R**	0.12 S	16 S	**64 R**
Campy spp	Site E	ng	ng	0.12 S	4 S	4 S	0.12 S	16 S	2 S
Campy spp	Site E	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	Site E	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	Site E	ng	2 S	0.25 S	2 S	1 S	0.5 S	**>64 R**	2 S
Campy spp	SP	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	SP	ng	4 I	0.25 S	4 S	0.5 S	ng	16 S	4 S

[A]Sample codes – CF (cattle feces), GF (geese feces), MG (microgreen), SF (swine feces), SP (sprout), Site C (river water), Site E (river water)
[B]AZI (azithromycin), CIP (ciprofloxacin), ERY (erythromycin), FFN (florfenicol), TET (tetracycline), GEN (gentamicin), NAL (nalidixic acid), CLI (clindamycin)
[C] Breakpoints were adopted from Clinical and Laboratory Standards Institute (CLSI) as reported in *(35)*, R (resistant), I (intermediate), S (susceptible).
[D]Florfenicol resistance breakpoint not established by CLSI, ≥8µg/mL established for resistance based upon NARMS reporting.
[E]No growth observed, indicting susceptibility to the lowest concentration of antibiotic tested AZI (0.015), CIP (0.015), CLI (0.03), ERY (0.03), FFN (0.03), TET (0.06), GEN (0.12), NAL (4)
[F]Bolded values indicate resistance.

Table 2.4. *Campylobacter* positive isolates antibiotic susceptibility according to the Clinical Laboratory Standards Institute (CLSI)

Antimicrobial Class	Antimicrobial Agent	Breakpoint MIC (µg/mL)		% (no.) *Campylobacter* spp.
Aminoglycosides	Gentamicin	S[A]	≤ 2	8 (3)
		I	4	3 (1)
		R	≥ 8	5 (2)
Lincosamides	Clindamycin	S	≤ 2	15 (6)
		I	4	15 (6)
		R	≥ 8	15 (6)
Macrolides	Azithromycin	S	≤ 2	46 (18)
		I	4	0 (0)
		R	≥ 8	3 (1)
	Erythromycin	S	≤ 8	36 (14)
		I	16	5 (2)
		R	≥ 32	8 (3)
Phenicols	Florfenicol	S	≤ 4	38 (15)
		I	N/A	0 (0)
		R	N/A[B]	10 (4)
Quinolones	Ciprofloxacin	S	≤ 1	33 (13)
		I	2	0 (0)
		R	≥ 4	5 (2)
	Nalidixic Acid	S	≤ 16	26 (10)
		I	32	3 (1)
		R	≥ 64	18 (7)
Tetracyclines	Tetracycline	S	≤ 4	31 (12)
		I	8	0 (0)
		R	≥ 16	15 (6)

[A]MIC breakpoints – susceptible (S), intermediate (I), resistant (R)
[B]Florfenicol resistance breakpoint not established by CLSI, ≥8µg/mL established for resistance based upon NARMS reporting.

Appendix C



Figure 3.1. Tennessee River Water Collection Sites A, B, C, and D

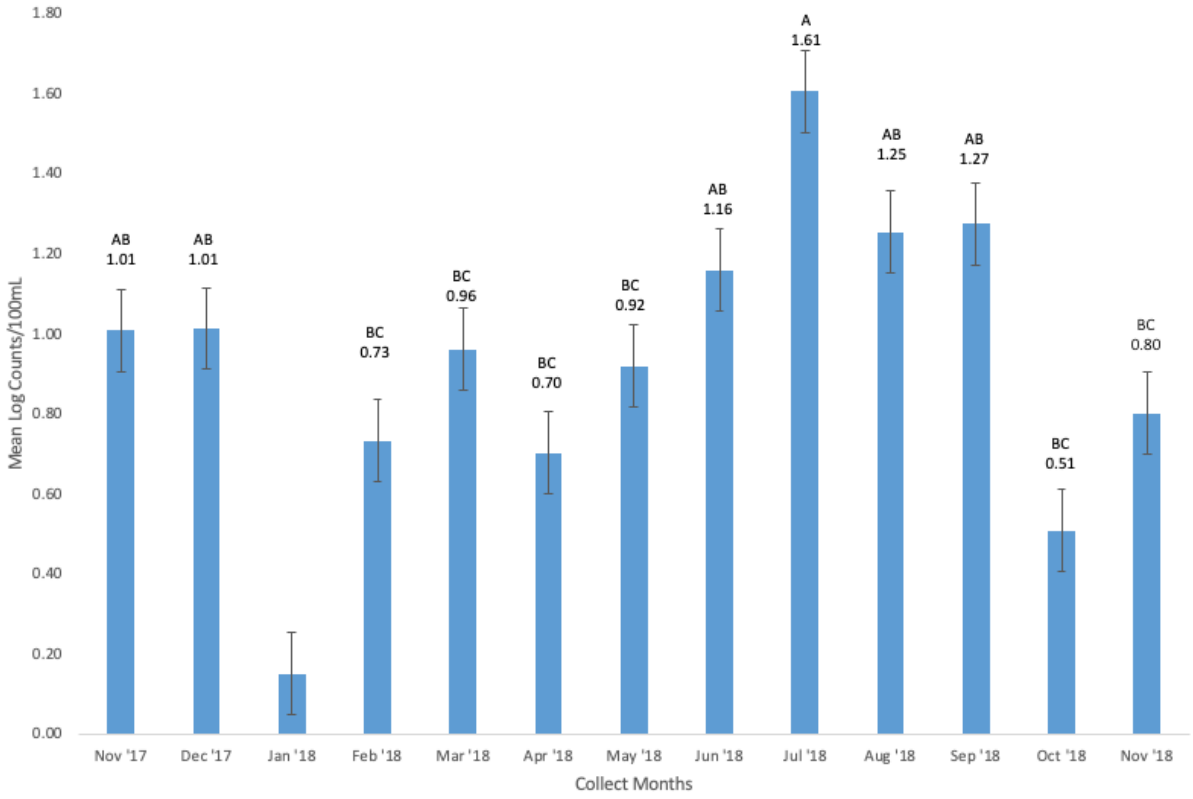

Figure 3.2. Mean separation of *Campylobacter* species log CFU/100mL at Site C from November 2017 to November 2018

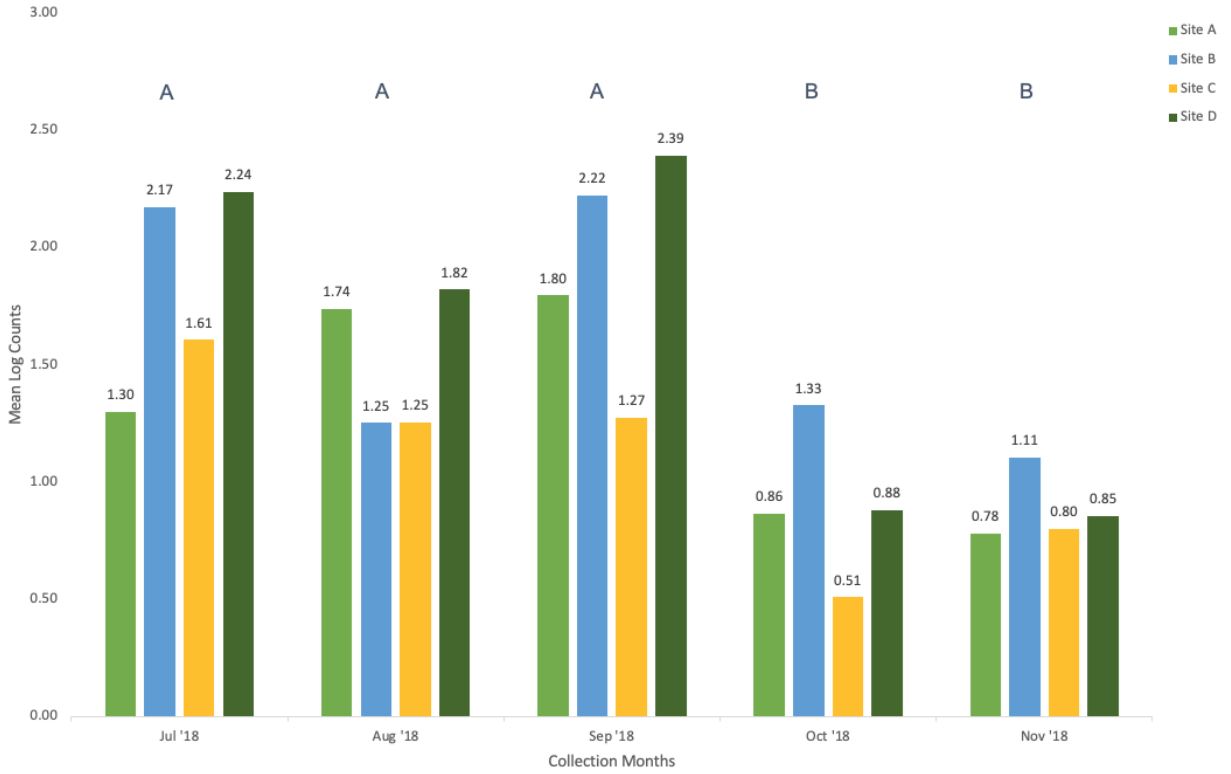

Figure 3.3. Mean separation of *Campylobacter* species log CFU/100mL at Sites A, B, C, and D from July 2018 to November 2018

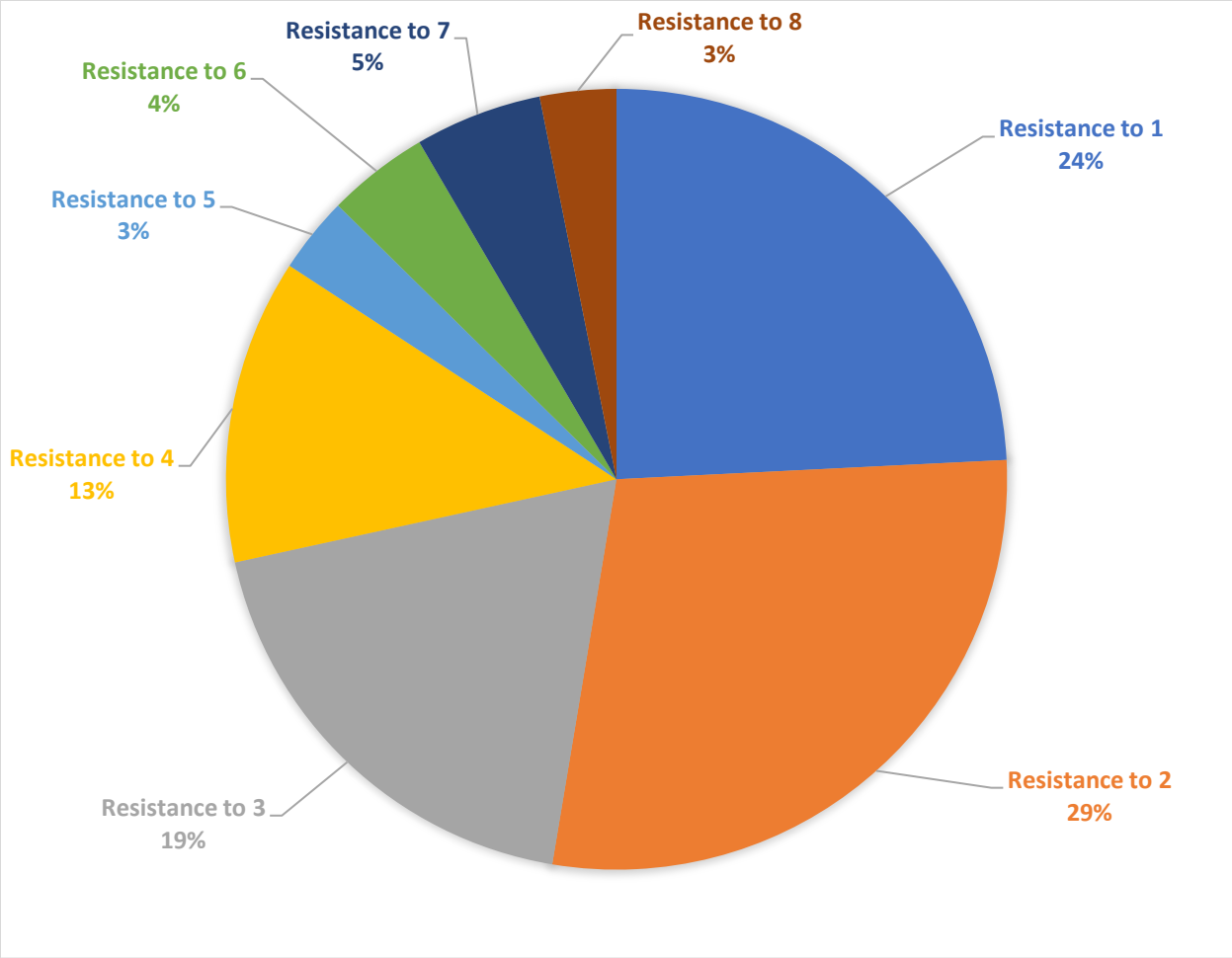

Figure 3.4. *Campylobacter* positive isolates expressing resistance as defined by Clinical and Laboratory Standards Institute (CLSI) to tested antibiotics on Sensitire CAMPY2 plates (n=95) (azithromycin, ciprofloxacin, erythromycin, florfenicol, tetracycline, gentamicin, nalidixic acid, clindamycin)

Table 3.1. PCR primer sequences selected from Saiyudthong et al, 2015 *(31)*

	Primer	Sequence (5’ to 3’)	Size (bp)
Campylobacter	Campy-F	GGA GGC AGC AGT AGG GAA TA	1,062
	Campy-R	TGA CGG GCG GTG AGT ACA AG	
C. jejuni	Jejuni-F	CAT CTT CCC TAG TCA AGC CT	773
	Jejuni-R	AAG ATA TGG CTC TAG CAA GAC	
C. coli	Coli-F	GGT ATG ATT TCT ACA AAG CGA G	502
	Coli-R	ATA AAA GAC TAT CGT CGC GTG	
C. lari	Lari-F	TAG AGA GAT AGC AAA AGA GA	251
	Lari-R	TAC ACA TAA TAA TCC CAC CC	

Table 3.2. PCR reaction well components

AmpliTaq Gold Fast PCR, UP	10 µL
Forward Primer	2 µL
Reverse Primer	2 µL
dH_2O	5 µL
Sample extracted DNA	1 µL

Table 3.3. Minimal Inhibitory Concentrations (MICs) of *Campylobacter* spp. confirmed samples using Sensitire CAMPY2 plates

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
C. coli	C	ng[E]	ng	ng	ng	ng	ng	ng	ng
C. coli	C	ng	**16 R[F]**	0.25 S	8 S	4 S	1 S	ng	2 S
C. coli	C	**8 R**	**8 R**	0.5 S	16 I	0.5 S	1 S	**>64 R**	2 S
C. coli	C	ng	ng	ng	ng	ng	ng	ng	ng
C. coli	C	ng	0.5 S	1 S	ng	ng	0.5 S	ng	ng
Campy spp	A	**>32 R**	**>16 R**	1 S	**64 R**	**>64 R**	**16 R**	**>64 R**	**>64 R**
Campy spp	A	ng	**>16 R**	2 S	**32 R**	**16 R**	0.12 S	32 I	4 S
Campy spp	A	ng	**>16 R**	4 I	**32 R**	**16 R**	0.25 S	**>64 R**	2 S
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	2 S	2 S	4 I	0.5 S	2 S	2 I	**>64 R**	1 S
Campy spp	A	0.5 S	**16 R**	2 S	**32 R**	**16 R**	ng	**>64 R**	2 S
Campy spp	A	**>32 R**	**>16 R**	4 I	**32 R**	**16 R**	0.12 S	32 I	2 S
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	**8 R**	**>16 R**	4 I	**32 R**	2 S	0.5 S	32 I	1 S
Campy spp	A	**8 R**	**>16 R**	4 I	4 S	**16 R**	1 S	**64 R**	4 S
Campy spp	A	ng	4 I	0.12 S	1 S	**8 R**	0.25 S	32 I	2 S
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	**16 R**	**>16 R**	**16 R**	**64 R**	**8 R**	**4 R**	**>64 R**	8 I
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng

Table 3.3. Continued

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	A	ng[E]	**16 R**	0.25 S	4 S	**32 R**	0.12 S	32 I	4 S
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	ng	ng	0.06 S	0.12 S	2 S	0.25 S	**>64 R**	0.5 S
Campy spp	A	0.5 S	4 I	1 S	4 S	1 S	1 S	**>64 R**	0.5 S
Campy spp	A	0.25 S	4 I	1 S	8 S	2 S	0.12 S	16 S	0.12 S
Campy spp	A	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	A	0.25 S	**>16 R**	1 S	16 I	4 S	0.25 S	32 I	**16 R**
Campy spp	A	0.5 S	4 I	2 S	4 S	1 S	0.5 S	**64 R**	0.5 S
Campy spp	A	0.25 S	2 S	2 S	4 S	2 S	0.5 S	**>64 R**	4 S
Campy spp	B	**>32 R**	**>16 R**	0.5 S	2 S	2 S	0.25 S	**64 R**	4 S
Campy spp	B	**>32 R**	**>16 R**	4 I	**32 R**	**8 R**	0.06 S	32 I	2 S
Campy spp	B	**>32 R**	**>16 R**	**16 R**	**64 R**	**8 R**	0.5 S	**>64 R**	4 S
Campy spp	B	0.25 S	**8 R**	0.25 S	1 S	2 S	0.12 S	**>64 R**	2 S
Campy spp	B	**>32 R**	**>16 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**
Campy spp	B	**>32 R**	**>16 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**
Campy spp	B	0.5 S	**>16 R**	**8 R**	**32 R**	**16 R**	0.12 S	**64 R**	2 S
Campy spp	B	2 S	4 I	0.5 S	4 S	4 S	**8 R**	**>64 R**	2 S
Campy spp	B	ng	4 I	0.25 S	4 S	4 S	0.12 S	**>64 R**	2 S
Campy spp	B	0.5 S	**>16 R**	2 S	16 I	**16 R**	0.03 S	**>64 R**	4 S
Campy spp	B	1 S	**16 R**	0.25 S	4 S	1 S	1 S	**>64 R**	4 S

Table 3.3 Continued

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	B	ng[E]	**8 R**	1 S	4 S	**16 R**	0.12 S	32 I	4 S
Campy spp	B	ng	**>16 R**	2 S	**32 R**	**16 R**	0.25 S	**>64 R**	4 S
Campy spp	B	ng	4 I	0.12 S	1 S	**8 R**	0.06 S	**64 R**	2 S
Campy spp	B	**8 R**	**>16 R**	2 S	**32 R**	**16 R**	1 S	**64 R**	**16 R**
Campy spp	B	1 S	**8 R**	0.06 S	2 S	4 S	0.12 S	**>64 R**	4 S
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	**16 R**	**>16 R**	4 I	**32 R**	**32 R**	**4 R**	**64 R**	8 I
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	2 S	**>16 R**	2 S	8 S	4 S	2 I	**>64 R**	**16 R**
Campy spp	B	0.25 S	**16 R**	0.5 S	4 S	**16 R**	0.12 S	16 S	2 S
Campy spp	B	1 S	4 I	0.5 S	8 S	2 S	0.12 S	8 S	0.25 S
Campy spp	B	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	B	1 S	**8 R**	2 S	16 I	2 S	0.25 S	32 I	0.25 S
Campy spp	B	0.5 S	2 S	4 I	4 S	2 S	1 S	**>64 R**	2 S
Campy spp	C	ng	**>16 R**	2 S	8 S	2 S	1 S	ng	8 I
Campy spp	C	**>32 R**	**>16 R**	2 S	8 S	4 S	2 I	32 I	8 I
Campy spp	C	**16 R**	**>16 R**	2 S	8 S	4 S	2 I	16 S	8 I
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	1 S

Table 3.3. Continued

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	C	**8 R**	**>16 R**	2 S	4 S	4 S	**4 R**	32 I	4 S
Campy spp	C	ng[E]	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	**8 R**	**8 R**	0.5 S	16 I	0.5 S	1 S	**>64 R**	4 S
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	0.25 S	**8 R**	1 S	16 I	4 S	0.25 S	**>64 R**	**64 R**
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	0.5 S	2 S	0.5 S	4 S	4 S	0.5 S	8 S	4 S
Campy spp	C	ng	2 S	2 S	2 S	1 S	0.5 S	**>64 R**	2 S
Campy spp	C	ng	4 I	0.25 S	4 S	2 S	0.12 S	32 I	1 S
Campy spp	C	4 I	1 S	0.03 S	2 S	1 S	1 S	**64 R**	2 S

Table 3.3 Continued

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	C	ng[E]	2 S	0.06 S	0.25 S	0.12 S	0.12 S	32 I	0.25 S
Campy spp	C	ng	1 S	0.03 S	0.25 S	1 S	0.12 S	32 I	2 S
Campy spp	C	0.5 S	**8 R**	0.12 S	1 S	4 S	0.25 S	**64 R**	4 S
Campy spp	C	0.25 S	**8 R**	0.12 S	1 S	4 S	0.12 S	32 I	4 S
Campy spp	C	0.25 S	**16 R**	0.25 S	2 S	4 S	0.25 S	**64 R**	2 S
Campy spp	C	0.12 S	**>16 R**	4 I	**>64 R**	**32 R**	0.25 S	**>64 R**	**>64 R**
Campy spp	C	0.25 S	4 I	1 S	2 S	1 S	1 S	32 I	0.5 S
Campy spp	C	4 I	**>16 R**	0.5 S	4 S	**32 R**	0.12 S	32 I	4 S
Campy spp	C	ng	2 S	0.25 S	1 S	2 S	1 S	**>64 R**	8 I
Campy spp	C	2 S	**>16 R**	1 S	4 S	2 S	0.5 S	**>64 R**	2 S
Campy spp	C	2 S	**>16 R**	0.5 S	8 S	4 S	0.5 S	**>64 R**	4 S
Campy spp	C	2 S	**8 R**	0.25 S	1 S	**8 R**	1 S	**>64 R**	8 I
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	**>32 R**	**>16 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**	**>64 R**
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	1 S	**>16 R**	0.5 S	8 S	1 S	0.12 S	32 I	1 S
Campy spp	C	0.5 S	**>16 R**	**>64 R**	16 I	**8 R**	0.12 S	**>64 R**	4 S
Campy spp	C	ng	**8 R**	0.25 S	2 S	**16 R**	0.12 S	16 S	4 S
Campy spp	C	ng	**>16 R**	4 I	**32 R**	**32 R**	0.12 S	32 I	4 S
Campy spp	C	ng	**16 R**	0.25 S	2 S	**8 R**	0.5 S	32 I	2 S
Campy spp	C	ng	**>16 R**	2 S	16 I	**16 R**	0.06 S	**>64 R**	4 S
Campy spp	C	ng	**>16 R**	2 S	**32 R**	**16 R**	0.03 S	**>64 R**	4 S
Campy spp	C	**>32 R**	**>16 R**	4 I	**32 R**	**16 R**	**32 R**	**>64 R**	**16 R**

Table 3.3 Continued

Isolate	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	C	0.5 S	**16 R**	1 S	2 S	1 S	0.03 S	**>64 R**	2 S
Campy spp	C	0.25 S	4 I	2 S	2 S	2 S	0.06 S	**>64 R**	1 S
Campy spp	C	ng[E]	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	**16 R**	0.12 S	4 S	**16 R**	0.12 S	32 I	4 S
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	**16 R**	0.5 S	4 S	**32 R**	0.25 S	**64 R**	**16 R**
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	0.5 S	**>16 R**	0.25 S	4 S	4 S	0.12 S	32 I	0.5 S
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	C	ng	4 I	0.25 S	4 S	**8 R**	0.12 S	32 I	4 S
Campy spp	C	2 S	**16 R**	4 I	**32 R**	4 S	0.5 S	**>64 R**	2 S
Campy spp	C	1 S	**8 R**	1 S	1 S	4 S	0.5 S	**>64 R**	0.25 S
Campy spp	C	ng	4 I	0.5 S	4 S	1 S	0.25 S	32 I	0.25 S
Campy spp	C	1 S	4 I	0.25 S	1 S	0.25 S	0.5 S	32 I	4 S
Campy spp	C	0.25 S	**>16 R**	2 S	8 S	4 S	0.5 S	**>64 R**	1 S
Campy spp	C	ng	**8 R**	0.5 S	1 S	0.25 S	0.5 S	**>64 R**	0.25 S
Campy spp	D	0.5 S	1 S	1 S	1 S	1 S	0.25 S	16 S	1 S
Campy spp	D	ng	**>16 R**	0.12 S	4 S	2 S	0.25 S	**>64 R**	8 I
Campy spp	D	1 S	1 S	0.12 S	1 S	1 S	0.12 S	**>64 R**	0.5 S

Table 3.3 Continued

Isolates	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	**Quinolones**		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	**NAL**	TET
Campy spp	D	1 S	2 S	0.12 S	1 S	4 S	0.5 S	**>64 R**	0.5 S
Campy spp	D	0.5 S	**>16 R**	**8 R**	**64 R**	**16 R**	2 I	**>64 R**	8 I
Campy spp	D	4 I	**16 R**	1 S	8 S	**16 R**	0.25 S	**>64 R**	2 S
Campy spp	D	0.5 S	**>16 R**	2 S	8 S	**64 R**	**4 R**	**>64 R**	8 I
Campy spp	D	**>32 R**	**>16 R**	**32 R**	**32 R**	**64 R**	**4 R**	32 I	**64 R**
Campy spp	D	ng[E]	ng	ng	ng	ng	ng	ng	ng
Campy spp	D	ng	**>16 R**	2 S	**32 R**	**16 R**	0.25 S	**>64 R**	4 S
Campy spp	D	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	D	**8 R**	4 I	0.5 S	4 S	2 S	0.25 S	**>64 R**	**16 R**
Campy spp	D	1 S	**8 R**	**>64 R**	2 S	0.25 S	1 S	**>64 R**	1 S
Campy spp	D	2 S	**16 R**	0.5 S	1 S	0.5 S	0.5 S	32 I	1 S
Campy spp	D	ng	**16 R**	0.25 S	2 S	**8 R**	0.12 S	**64 R**	4 S
Campy spp	D	0.5 S	ng	0.25 S	0.5 S	0.5 S	**8 R**	**>64 R**	1 S
Campy spp	D	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	D	ng	ng	ng	ng	ng	ng	ng	ng
Campy spp	D	**>32 R**	**>16 R**	**16 R**	**>64 R**	**32 R**	0.06 S	**64 R**	4 S
Campy spp	D	**>32 R**	**>16 R**	2 S	16 I	**16 R**	2 I	**>64 R**	8 I
Campy spp	D	4 I	**>16 R**	0.5 S	8 S	4 S	1 S	**>64 R**	**16 R**
Campy spp	D	**>32 R**	**>16 R**	4 I	**32 R**	**16 R**	**8 R**	**>64 R**	**64 R**
Campy spp	D	1 S	**>16 R**	0.12 S	8 S	4 S	0.5 S	**64 R**	4 S
Campy spp	D	1 S	**16 R**	2 S	8 S	2 S	2 I	32 I	0.5 S
Campy spp	D	0.5 S	4 I	0.5 S	2 S	1 S	0.12 S	32 I	0.12 S
Campy spp	D	1 S	**8 R**	4 I	8 S	4 S	0.5 S	**64 R**	2 S

Table 3.3 Continued

Isolates	Collection Site[A]	MIC (µg/mL)[B] and Antibiotic Susceptibility[C] (Drug Class)							
		Aminoglycoside	Lincosamide	Macrolides		Phenicol	Quinolones		Tetracycline
		GEN	CLI	AZI	ERY	FFN[D]	CIP	NAL	TET
Campy spp	D	0.5 S	1 S	ng[E]	0.25 S	0.25 S	ng	32 I	4 S
Campy spp	D	1 S	4 I	0.25 S	2 S	1 S	1 S	**>64 R**	1 S
Campy spp	D	0.5 S	**8 R**	2 S	16 I	1 S	0.5 S	**>64 R**	2 S

[A]Sample codes – A (river water), B (river water), C (river water), Site D (river water)
[B]AZI (azithromycin), CIP (ciprofloxacin), ERY (erythromycin), FFN (florfenicol), TET (tetracycline), GEN (gentamicin), NAL (nalidixic acid), CLI (clindamycin)
[C] Breakpoints were adopted from Clinical and Laboratory Standards Institute (CLSI) as reported in *(37)*, R (resistant), I (intermediate), S (susceptible).
[D]Florfenicol resistance breakpoint not established by CLSI, ≥8µg/mL established for resistance based upon NARMS reporting.
[E]No growth observed, indicting susceptibility to the lowest concentration of antibiotic tested AZI (0.015), CIP (0.015), CLI (0.03), ERY (0.03), FFN (0.03), TET (0.06), GEN (0.12), NAL (4)
[F]Bolded values indicate resistance.

Table 3.4. *Campylobacter* positive isolates antibiotic susceptibility according to the Clinical and Laboratory Standards Institute (CLSI)

Antimicrobial Class	Antimicrobial Agent	Breakpoint MIC (µg/mL)		% (no.) *Campylobacter* spp.
Aminoglycosides	Gentamicin	S[A]	≤ 2	32 (52)
		I	4	2 (4)
		R	≥ 8	14 (23)
Lincosamides	Clindamycin	S	≤ 2	9 (14)
		I	4	10 (17)
		R	≥ 8	47 (75)
Macrolides	Azithromycin	S	≤ 2	51 (82)
		I	4	9 (14)
		R	≥ 8	7 (11)
	Erythromycin	S	≤ 8	44 (71)
		I	16	6 (10)
		R	≥ 32	16 (26)
Phenicols	Florfenicol	S	≤ 4	39 (63)
		I	N/A	0 (0)
		R	N/A[B]	27 (44)
Quinolones	Ciprofloxacin	S	≤ 1	54 (87)
		I	2	4 (6)
		R	≥ 4	8 (13)
	Nalidixic acid	S	≤ 16	4 (7)
		I	32	18 (29)
		R	≥ 64	43 (70)
Tetracyclines	Tetracycline	S	≤ 4	52 (84)
		I	8	6 (9)
		R	≥ 16	9 (15)

[A]MIC breakpoints – susceptible (S), intermediate, (I), resistant (R)
[B]Florfenicol resistance breakpoint not established by CLSI, ≥8µg/mL established for resistance based upon NARMS reporting.

Vita

Molly Albin West was born in Memphis, Tennessee, on June 10, 1991. Molly grew up outside Memphis and graduated from Houston High School in 2009. Like her family, she continued her education by attending The University of Tennessee, Knoxville, where she earned a B.S. in Food Science and Technology with a concentration in science. Upon culmination of her undergraduate studies, Molly earned a Master of Science in Food Science and a certificate in Food Safety with an emphasis in Food Safety Education. Following an internship in public health, she began a Doctoral program at The University of Tennessee in Food Microbiology. Her parents, David and Pamela Albin always encouraged her and her sister to never stop what they start. This was never truer than during her doctoral studies. Upon earning her Ph.D. and minor in Epidemiology, Molly will pursue a career in academia where she can continue her passion for extension education.