ABSTRACT

Title: ***CAMPYLOBACTER* SPP. IN BULK TANK MILK AND MILK FILTERS FROM US DAIRY FARMS**

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Campylobacter spp. are a common cause of foodborne outbreaks associated with raw or unpasteurized milk, and *Campylobacter* spp. have also been detected on most dairies in the US. An estimate of the prevalence of thermophilic *Campylobacter* spp. in bulk tank milk (BTM) on US dairy operations was determined as part of the National Animal Health Monitoring System's Dairy 2014 study. *Campylobacter* spp. were detected in the BTM and milk filters from 34.2% of the 234 dairies. Isolates were obtained from 18.4% of the dairies. *C. jejuni* was the most frequently isolated species, and this species is also the most common cause of human infection. When resistance to a panel of nine antimicrobials was tested, 68.4% of *C. jejuni* isolates were resistant to tetracycline. This survey suggests that BTM from US dairies can be contaminated with pathogenic *Campylobacter* spp., and the consumption of unpasteurized, raw milk represents a human health risk.

CAMPYLOBACTER SPP. IN BULK TANK MILK AND MILK FILTERS FROM US DAIRY FARMS

By

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Chapter 1: Introduction

Campylobacter spp. are small, spiral and motile gram-negative bacteria that are approximately 0.2 to 0.8 µm wide and 0.5 to 5 µm long and reside in the gastrointestinal tract of many warm-blooded animals (Debruyne et al., 2008). There are currently between 16 and 20 recognized *Campylobacter* species as well as multiple subspecies and biovars. *Campylobacter* was first described by Theodor Escherich in 1886 and was later identified in sheep in 1906 and in aborted bovine fetuses in 1913 (Debruyne et al., 2008). Following improvements in culture techniques, *Campylobacter* spp. were also recognized as a human pathogen in the late twentieth century. *Campylobacter* spp. are now a leading bacterial cause of human gastrointestinal illness in both the developing and developed world. In the US, *Campylobacter* spp. infections have increased by 13% since 2006, and the Centers for Disease Control and Prevention (CDC) estimates that there were 13.45 infections per 100,000 people in 2014 (CDC, 2015).

1.1 Human Illness

Infection in humans that is caused by *Campylobacter* spp. is known as campylobacteriosis, and it is characterized by an acute inflammatory lesion, primarily originating in the jejunum and ileum. *C. jejuni* is the primary cause of laboratory-confirmed infections, and *C. coli* is the second most commonly isolated species (CDC, 2015). Other laboratory-confirmed species that cause campylobacteriosis in the US include *C. lari, C. upsaliensis, C. fetus, C. hyointestinalis, C. sputorum, C. helveticus, C. gracilis, C. muscosalis,* and *C.*

showae (CDC, 2014; CDC, 2015). The infective dose may be as low as 500 cells (Black et al., 1988; Robinson, 1981). After an average incubation period of 3.2 days, *Campylobacter* spp. infection generally elicits cramping pains followed by diarrhea, nausea, and continued abdominal pain. Symptoms are usually acute and begin to resolve after about 3 to 4 days; however, *Campylobacter* spp. may be shed in patients' feces for several weeks.

While infection is usually self-limiting, antibiotics may be prescribed. When necessary, the antibiotics of choice are macrolides, such as erythromycin (Blaser and Engberg, 2008). Until recently, fluoroquinolones, such as ciprofloxacin, have also been used as an alternative; however, the most recent national survey of antimicrobial resistance in the US identified resistance to ciprofloxacin in 22% of human *C. jejuni* isolates (CDC NARMS, 2015). Alternative antibiotics used to treat campylobacteriosis include tetracycline, doxycycline and chloramphenicol.

Although gastrointestinal illness caused by *Campylobacter* spp. may resolve with or without antimicrobial intervention, several post-infection sequelae can develop weeks after the initial onset of infection. *Campylobacter* spp. have been identified as the source for approximately 20-50% of cases of Guillain-Barré syndrome, a peripheral neuropathy caused by molecular mimicry (Jacobs et al., 2008). Lipooligosaccharides on the surface of *Campylobacter* cells resemble human neuronal gangliosides, leading to an autoimmune response against the nerves. Reactive arthritis has also been identified as a post-infection sequela, with incidence rates estimated to be between 1% and 5% of individuals previously

infected by *Campylobacter* spp. (Pope et al., 2007). Reactive arthritis may develop within 4 weeks of gastrointestinal illness caused by *Campylobacter* spp., as well as by other pathogens such as *Salmonella* and *Shigella*.

1.2 Metabolism and Pathogenesis

Campylobacter is a microaerophilic organism that requires 5-10% oxygen, and its ideal growth temperature is 30-37°C. Several species, such as *C. hyointestinalis*, also require hydrogen for growth (Han et al., 1991). Thermophilic species, such as *C. jejuni*, will not grow below 30°C, and they thrive at 42°C. *Campylobacter* spp. cannot ferment or oxidize carbohydrate due to a lack of both 6-phosphofructokinase and an active phospheoenolpyruvate-dependent phosphotransferase system (Velayudhan and Kelly, 2002). Rather, *Campylobacter* spp. obtain carbon from the oxidation of amino acids and tricarboxylic acids. *Campylobacter* spp. are very sensitive to many environmental conditions, such as drying or freezing (Cox et al., 2001). *Campylobacter* spp. require an elevated water activity level (0.987 to 0.997) and cannot withstand temperature fluctuations (Silva et al., 2011). During periods of stress or aging, *Campylobacter* spp. may become coccoid (Ng et al., 1985). In the laboratory setting, *Campylobacter* spp. must be provided a suitable atmospheric environment with appropriate sources of nutrients. For instance, *Campylobacter* spp. must be grown in a microaerophilic environment of 5% oxygen. Several less common, emerging species, such as *C. hyointestinalis* and *C. sputorum*, require hydrogen enrichment as well. Furthermore, an elevated incubation temperature of 42°C will preferentially select for the more common thermophilic species, *C. jejuni, C. coli*

and *C. lari*, while less thermotolerant species, such as *C. fetus*, may fail to grow. Additionally, antimicrobial supplementation, which is commonly added to isolation media to inhibit competition of other enteric flora, may also inhibit the growth of *Campylobacter* spp. that are not resistant to cefoperazone, including *C. concisus*. Therefore, *Campylobacter* spp. are best suited for the gastrointestinal environment of warm-blooded animals, and isolation in the laboratory setting is dependent upon the specific materials and methods that are used.

The pathogenesis of *Campylobacter* spp. is not as well-defined as other foodborne pathogens. However, some details are known about the colonization and invasion mechanisms and the varied pathogenicity in different host species. In the chicken gut, *Campylobacter* spp. primarily colonize the deep crypts of the cecum at up to 10^{10} colony-forming units per gram (Young et al., 2007). In bovine, *Campylobacter* spp. preferentially colonize the small and large intestine and are absent from the rumen (Inglis et al., 2005; Krueger et al., 2008), and *Campylobacter* spp. colonize the small intestine in humans.

Several virulence factors have been identified as important for *Campylobacter* spp. survival and colonization. Motility via one or two polar flagella is vital to colonization of intestinal cells (Hermans et al., 2011). The corkscrew motion first allows penetration of the gut mucus layer. The flagella also act as a type III secretion system or flagellar export system which injects effector proteins, or *Campylobacter* invasion antigens (Cia), into the enterocyte (Konkel et al., 2004). The outer membrane protein, CadF, also binds to fibronectin on the basolateral epithelial cells of the gut to facilitate adhesion and

invasion (Konkel et al., 2001; Muller et al., 2006). *Campylobacter* spp. cells secrete cytolethal distending toxin (CDT), which cause cell cycle arrest and the eventual cell death of enterocytes (Young et al., 2007). The toxin is composed of subunits CdtA and CdtC, as well as the active subunit, CdtB. In the nucleus, CdtB exhibits a DNase-like activity. Death of enterocyte cells is also due to cytoskeletal rearrangements which inevitably lead to a loss of cell function and compromised barrier.

1.3 Foodborne Illness

1.3.1 Foodborne Outbreaks.

In the US, foodborne illnesses are actively monitored by the CDC's Foodborne Diseases Active Surveillance Network (FoodNet). According to FoodNet and the CDC, *Campylobacter* spp. infections have increased by 13% since 2006, leading to about 1.3 million cases and 120 deaths each year (Crim et al., 2015). Most sporadic illnesses are due to contaminated poultry; however, outbreaks have frequently been attributed to dairy products (Taylor et al., 2013; CDC, 2014). Between 1978 and 1986, 57.8% of foodborne outbreaks were linked to raw milk (Tauxe, 1992). The single most identified source of foodborne *Campylobacter* spp. outbreaks between 1997 and 2008 was due to the consumption of raw dairy products (28.0%, Taylor et al., 2013). Langer (2012) and Mungai (2015) also analyzed milk-related outbreak data from 1993 to 2012 and found that the majority of foodborne outbreaks associated with raw milk were due to *Campylobacter* spp. Outbreaks caused by raw milk or contaminated water have been shown to have bimodal distribution, with more outbreaks observed in

May and October, possibly due to seasonal shedding of *Campylobacter* spp. in the feces of dairy cattle (Tauxe, 1992).

1.3.2 Prevalence in Animals.

Campylobacter spp. are considered to be commensal organisms of many wild animals as well as food animals, including poultry, cattle, sheep, and swine species. *C. jejuni* is the primary species found in poultry and cattle while *C. coli* is the most common *Campylobacter* spp. in swine (Manser and Dalziel, 1985). Other bovine-associated species include *C. hyointestinalis, C. fetus* subsp. *fetus,* and *C. lanianae.* Although most notably present in poultry intended for human consumption, such as chickens and turkeys, *Campylobacter* spp. have also been detected in wild birds, including migrating birds, which may be a source of transmission to farms (Sanad et al., 2013; Keller and Shriver, 2014; Ryu et al., 2014).

Previous surveys have found *Campylobacter* spp. to be present on most US dairy farms (USDA APHIS, 2011). Young calves excrete *Campylobacter* cells in high number from underdeveloped gastrointestinal systems while adults may shed intermittently throughout their lives (Nielson, 2002; Stanley and Jones, 2003). Dairy cattle feces may yield between 2.1- 2.8 $\log_{10}$ (most probable number, MPN) per gram (Stanley et al., 1998; Nielson, 2002). Generally, *Campylobacter* spp. are non-pathogenic commensals of the bovine intestines, with several notable exceptions. Although uncommon, *Campylobacter* spp. have occasionally been implicated in cases of mastitis (Hutchinson et al., 1985; Morgan et al., 1985; Orr et al., 1995; Bianchini et al., 2014). More recently, a highly pathogenic,

tetracycline-resistant *C. jejuni* strain, clone SA, has been identified as the cause of ruminant abortions (Sahin et al., 2012). *Campylobacter fetus* subsp. *fetus* also may cause abortions in cattle.

1.5 Milk and Pasteurization

Milk is a common beverage consumed by more than 6 billion people worldwide (Visioli and Strata, 2014). In the US, milk produced by dairy cows is the most accessible type of animal milk, with approximately 206,046,000 lbs of dairy milk produced in 2014 (USDA ERS, 2015). In addition to *Campylobacter* spp., unpasteurized, raw milk can harbor many other human pathogens, including gram-negative bacteria such as *Salmonella* spp., Shiga toxin-producing *Escherichia coli,* and *Yersinia* spp., and gram-positive bacteria, such as *Listeria* spp. and *Staphylococcus* spp. (Karns et al., 2007; Latorre et al., 2011; Van Kessel et al., 2011). These pathogens are known to cause mild to severe gastrointestinal illness with occasional severe sequelae and potentially death. Several surveys determined that the prevalence of common gastrointestinal pathogens in bulk tank milk (BTM) ranges from 0.2% to 32.1% (Doyle and Roman, 1982; Lovett et al. 1983; McManus and Lanier, 1987; Rohrbach et al., 1992; Jayarao and Henning, 2001; Jayarao et al., 2006; Van Kessel et al., 2008; Latorre et al., 2011; Van Kessel et al., 2011). The bulk tank is the final collection point at which milk is combined and stored from multiple cows; therefore, sampling BTM yields a farm prevalence pooled from the animals on the farm. Most BTM contamination is the result of fecal contamination, poor equipment hygiene or human handling. Risk factors for BTM contamination may include animal hygiene, the age and cleaning

frequency of milking equipment, pre-and post-udder treatment and milk storage (Cerva et al., 2014). Mastitis or asymptomatic shedding occasionally may also be sources of pathogens in BTM. Human pathogenic organisms that also cause mastitis include *Staphylococcus aureus, E. coli, Streptococcus agalactiae*, *S. dysgalactiae*, *S. uberis*, and occasionally, *Salmonella, Listeria monocytogenes, Mycobacterium bovis, Mycobacterium tuberculosis,* and *C. jejuni* (Adams, 2008).

Several regional surveys have specifically determined the prevalence of *Campylobacter* spp. in BTM. Surveys of dairy farms in Minnesota/South Dakota and Pennsylvania isolated *Campylobacter* spp. from 9.2% and 2.0% of BTM samples, respectively (Jayarao and Henning, 2001; Jayarao et al., 2006). Previous work with dairy-associated pathogens has also revealed the value of milk filter sampling as a more sensitive method of detecting of bacterial pathogens than BTM sampling alone (Van Kessel et al., 2008; Latorre et al., 2011; Van Kessel et al., 2011). The purpose of the milk filter is to remove large debris, such as bedding or feces, from the milk prior to storage in the bulk tank. With a common pore size of between 100 and 250 μm, the filter is not designed to remove bacterial pathogens; however, bacterial cells may concentrate on the filter along with the debris. For example, a multi-state survey of 128 organic and conventional farms in the Michigan, Minnesota, New York, and Wisconsin obtained eleven *Campylobacter* spp. isolates from milk filters while three isolates were obtained from matching BTM samples (Halbert et al., 2006).

Although dairy operations employ several strategies to reduce bacterial contamination in milk, these strategies are not 100% effective. Pasteurization was

introduced in the early 20th century as a method of combating diphtheria and typhoid fever. Low temperature-long time (LTLT) pasteurization involves heating at 63°C for 30 minutes, which is sufficient for killing *M. tuberculosis* and *Coxiella burnetti*, two heat-resistant non-spore-forming pathogens (Jay et al., 2005). The more commonly utilized high temperature-short time (HTST) method involves heating to 72°C for 15 seconds. Ultra-high temperature (UHT) pasteurization requires heating for 135-140°C for at least 1 second. Pasteurization is also commonly coupled with homogenization, which breaks down fat globules in the milk.

Although pasteurization greatly reduces the risk of foodborne illness, raw milk advocates have promoted potential health benefits and superior quality of unpasteurized, raw milk. Approximately 0.5 to 3.5% of the US population is estimated to drink raw milk (Lejeune and Rajala-Schultz, 2009). Proponents of drinking raw milk cite reductions in lactose intolerance and in the development of allergies as well as an improvement in taste and protein content (Davis et al., 2014). Current research does not support the claim that raw milk consumption may reduce lactose intolerance. While several studies have observed a connection between raw milk and the prevention of allergies, authors of these studies still caution against consuming raw milk, citing the risk of illness due to zoonotic pathogens. Safer alternatives with a reduced risk of illness that also maintain many qualities of raw milk include vat pasteurization and non-homogenized milk. Additionally, many of the nutrients found in raw milk can be acquired from much safer food sources in a well-balanced diet. Due to the clear risk of foodborne

illness and lack of scientific evidence supporting beneficial claims, several major organizations, including the American Academy of Pediatrics and the National Mastitis Council, have issued position statements advising against the consumption of raw milk.

Although alternatives exist and the transport of raw milk between states has been banned by the Food and Drug Administration (FDA) since 1987 (21 C.F.R. § 1240.61) due to health concerns, some form of raw milk sales is permitted within 42 states (NCSL, 2015). Because intrastate sales are regulated by the individual states, several legal distribution methods exist. In the majority of states where it is legal, raw milk may be sold to consumers in retail stores, farmers' markets or on the farm. Other states, such as Ohio and Indiana, permit herd or cow shares, in which the consumer shares ownership of the cow or herd. In Arkansas, Kentucky and Mississippi, raw cows' milk sales are illegal; however, raw goats' milk may be sold. With the exception of Michigan, all US states permit raw milk sales for animal consumption, which is regulated under commercial feed licensing laws. The regulations imposed by the FDA that require pasteurization of milk sold between states also set forth guidelines for the processing of cheese made from raw milk. The sale of hard cheese made from raw milk is permitted provided that it has been processed for at least 60 days. Although outbreaks have been attributed to the consumption of cheese made from raw milk, they are far fewer than those attributed to the consumption of raw milk itself. The extended processing time of cheese results in a dry, acidic environment which generally inhibits the growth of pathogenic bacteria (Taylor et al., 2013).

Therefore, consumption of raw milk is the primary source of foodborne illness attributed to dairy products.

1.6 Antimicrobial Resistance

Due to increasing concern regarding the resistance of foodborne pathogens to antimicrobials used in human medicine, the CDC, along with the FDA, USDA and state and local public health departments, established an antimicrobial resistance monitoring system in 1996. The collaborative National Antimicrobial Resistance Monitoring System (NARMS) tracks antimicrobial resistance in enteric bacteria associated with human illness, food production animals and retail meats. Resistance is monitored in two pathogenic species, *Salmonella* spp. and *Campylobacter* spp., as well as two indicator species, *E. coli* and *Enterococcus* spp. Approximately 80% of *Salmonella* isolates were susceptible to all antimicrobials tested in 2012 and 2013 (CDC NARMS, 2014). Resistance to antimicrobials was noted most frequently in turkey isolates while cattle isolates were least frequently resistant. Similarly, resistance to at least one antimicrobial was more common in turkey *E. coli* isolates (~80%) and least common in cattle *E. coli* isolates (~20%). The majority of *Enterococcus* spp. isolates (90%) were resistant to at least one antimicrobial, with poultry isolates more frequently resistant than either cattle or swine. In 2012 and 2013, less than 3% of *C. jejuni* isolates from poultry and cattle were resistant to erythromycin, the drug of choice to treat campylobacteriosis in humans. Only 0.4% of dairy isolates were resistant to erythromycin. In comparison, 8.4% of dairy isolates were resistant to ciprofloxacin, an alternative campylobacteriosis treatment.

The NARMS program specifically monitors the resistance of zoonotic pathogens to clinically important antimicrobials, including fluoroquinolones, cephalosporins and macrolides. In 2013, 3% of nontyphoidal *Salmonella* isolates from humans were resistant to nalidixic acid, a precursor to the fluoroquinolone, ciprofloxacin (CDC NARMS, 2015). Additionally, 3% of *Salmonella* human isolates were resistant to ceftriaxone, a third-generation cephalosporin. Resistance to ciprofloxacin was detected in 22% of *C. jejuni* human isolates while resistance to each of two macrolides, erythromycin and azithromycin, was detected in 2% of isolates.

While NARMS and FoodNet monitor zoonotic pathogens in order to protect human health, the National Animal Health Monitoring System (NAHMS), which is coordinated by the USDA, monitors the health, management and productivity of domestic livestock in the US. The dairy industry has previously been surveyed by the NAHMS program in 1992, 1996, 2002, and 2007. During the NAHMS Dairy 2002 and 2007 studies, 49.5% and 36.6% of *C. jejuni* isolates were pansusceptible, respectively (Englen et al., 2007; USDA APHIS, 2011). In 2002, 46.9% of isolates were resistant to a single antimicrobial while 61.2% were resistant in 2007. Multidrug resistance was detected in 3.6% and 2.2% of isolates in 2002 and 2007. Fewer than 5% of isolates were resistance to azithromycin, ciprofloxacin, chloramphenicol, clindamycin, erythromycin, gentamicin, or nalidixic acid; however, 47.4% and 62.9% of isolates in 2002 and 2007 were resistant to tetracycline. Resistance to ciprofloxacin and erythromycin is particularly concerning due to their use in the treatment of human campylobacteriosis. Resistance to tetracycline is not unexpected due to its

common use in calf milk replacers and foot baths as well as for therapeutic purposes (Zwald et al., 2004; Sato et al., 2004; Halbert et al., 2006; USDA APHIS, 2011). Recently, Sahin et al. (2012) identified a highly pathogenic tetracycline-resistance strain of *C. jejuni* in ruminant reservoirs that is responsible for sheep and cattle abortions as well as human illness. Other surveys of fecal, milk and milk filter isolates in the US have also identified moderate resistance to lincomycin, kanamycin and sulfamethoxazole (Harvey et al., 2004; Halbert et al., 2006). Therefore, *Campylobacter* spp. isolated from dairy cows may also represent a reservoir for antimicrobial resistance.

1.7 Goals and Objectives

The goal of this research project was to assess the prevalence of the zoonotic pathogen, *Campylobacter*, in bulk tank milk and milk filters from US dairy operations. The following objectives were set in this study:

- To determine the prevalence and antimicrobial resistance of *Campylobacter* spp. isolated from US dairy operations.
- To identify *Campylobacter* species via multiplex PCR and to characterize and compare the *Campylobacter* spp. isolates via pulsed-field gel electrophoresis (PFGE).

Chapter 2: *Campylobacter* spp. in bulk tank milk and milk filters from US dairy farms

2.1 Abstract

Campylobacter is frequently isolated from dairy cows as commensal organisms. While sporadic *Campylobacter* infections in humans are generally attributed to poultry, outbreaks (defined as 2 or more affected people) are commonly associated with dairy products, particularly unpasteurized or raw milk. Bulk tank milk (BTM) samples and milk filters from US dairy operations were collected during the National Animal Health Monitoring System's Dairy 2014 study and analyzed by real-time PCR and traditional culture techniques for the presence of thermophilic *Campylobacter* species. *Campylobacter* spp. were detected in samples from 34.3% of dairy operations and isolated from 17.8% of operations. The majority (91.8%) of isolates were identified as *C. jejuni*, while *C. lari* and *C. coli* were also isolated. Resistance to tetracycline was detected in 68.4% of *C. jejuni* isolates while resistance to ciprofloxacin and nalidixic acid was detected 13.2% of isolates. Based on analysis of pulsed-field gel electrophoresis patterns, dairy-associated *C. jejuni* are geographically diverse. Several isolates from multiple states were identified as a tetracycline-resistant strain known for its virulence in ruminants and as a source of human infection. These results from the first national survey of BTM and milk filters suggest that BTM can commonly be contaminated with pathogenic *Campylobacter* spp. and that the consumption of unpasteurized or raw milk may present a potential health risk.

2.2 Introduction

Campylobacter spp. are microaerophilic, spiral-shaped gram-negative bacteria that are a leading cause of bacterial diarrhea in the US and account for at least 1 million cases each year (CDC, 2015). Campylobacteriosis is usually self-limiting, with symptoms such as mild diarrhea and cramping; however, severe cases may require antibiotics. Post-infection sequelae, such as Guillain-Barré syndrome, may cause life-long neurological problems. According to the Centers for Disease Control and Prevention (CDC), *Campylobacter* spp. infections in humans have increased by 13% from 2006 to 2014 (CDC, 2015). *C. jejuni* is the most common cause of campylobacteriosis; however, other thermophilic species, such as *C. coli* and *C. lari*, as well as emerging species, such as *C. fetus*, have also been known to cause illness (CDC, 2015; Taylor et al., 2013).

Although sporadic *Campylobacter* spp. infections are commonly associated with poultry, *Campylobacter* spp. outbreaks have also been attributed to the consumption of other foods, particularly unpasteurized or raw milk. Raw milk represents a small percent of total milk consumed in the US; however, increasing interest in the potential health benefits, due to not heating the milk, represents a growing risk for campylobacteriosis (Lejeune and Rajala-Schultz, 2009). Overall, the incidence of bacterial outbreaks involving raw milk has been estimated to be over 150 times greater than the incidence involving pasteurized milk (Langer et al., 2012). Based on a review of CDC data from 1997 to 2008, 28% of foodborne *Campylobacter* spp. outbreaks were due to the consumption of contaminated raw milk products, which represented the largest single identified

source of campylobacteriosis (Taylor et al., 2013). Langer (2012) and Mungai (2015) analyzed milk-related outbreak data from 1993 to 2012 and found that the majority of foodborne outbreaks associated with unpasteurized milk were due to *Campylobacter* spp. contamination. Dairy products, particularly unpasteurized milk, are an important cause of *Campylobacter* spp. infection.

Campylobacter spp. can colonize and be shed by otherwise healthy animals, including birds and mammals (Manser and Dalziel, 1985; Wesley et al., 2000; Nielson, 2002; Miller, 2005). Although *Campylobacter* spp. can been excreted in the milk of infected cows, this is an uncommon occurrence (Hutchinson et al., 1985; Morgan et al., 1985; Orr et al., 1995; Bianchini et al., 2014), and the presence of *Campylobacter* spp. in bulk tank milk (BTM) is usually due to fecal contamination from asymptomatic animals (Oliver et al., 2005). National and regional surveys have consistently identified *Campylobacter* spp. on US dairy farms, particularly in fecal samples. As part of the USDA-APHIS National Animal Health Monitoring System (NAHMS) Dairy studies in 1996, 2002 and 2007, *Campylobacter* was identified in the feces of cows from 90-100% of tested operations, and the most commonly isolated species was *C. jejuni*. (USDA APHIS, 2011). Regional and multi-state surveys have also identified *Campylobacter* spp. in BTM on US dairy farms. Surveys of farms between 1982 and 1992 isolated *Campylobacter* from 0.4% to 12.3% of BTM samples (Doyle and Roman, 1982; Lovett et al., 1983; McManus and Lanier, 1987; Rohrbach et al., 1992). These studies collectively surveyed farms in Kentucky, Ohio, Tennessee, Indiana, Illinois, Michigan, Wisconsin, and Virginia. More recently,

Jayarao isolated *Campylobacter* spp. from 9.2% of BTM samples from farms in Minnesota and South Dakota (Jayarao et al., 2001) and from 2.0% of BTM from farms in Pennsylvania (Jayarao et al., 2006). Therefore, *Campylobacter* spp. can clearly contaminate the BTM, despite farm management practices.

An additional concern for *Campylobacter* spp. as a foodborne pathogen is the potential for antimicrobial resistance. In the NAHMS Dairy Studies in 2002 and 2007, *C. jejuni* isolates were tested for resistance to a panel of 10 antimicrobials. Resistance to tetracycline was detected in 62.4% of *C. jejuni* isolates, compared to 47.5% in the 2002 study (USDA APHIS, 2011; Englen et al., 2007). Resistance to any of the 9 remaining antimicrobials ranged from 0% (susceptible) to 4.0%. Sahin et al. (2012) identified a highly pathogenic, tetracycline-resistant *C. jejuni* strain that was isolated from dairy farm environments. This strain, clone SA, has been implicated in ruminant abortions and has been isolated from human outbreaks attributed to raw milk as well as from raw milk and ruminant fecal samples. Other large dairy surveys have also reported *Campylobacter* spp. isolates with moderate resistance to multiple antibiotics, including kanamycin, lincomycin and sulfamethoxazole (Harvey et al., 2004; Halbert et al., 2006).

Although the interstate transport of raw milk has been banned by the FDA since 1987, intrastate sale is regulated by individual states. Currently, some form of raw milk distribution is permitted in 42 states (NCSL 2015). Raw milk is also commonly consumed by farm families and employees (Shiferaw et al., 2000; Hegarty et al., 2002; Jayarao et al., 2006). Despite detection in both feces and

BTM from US dairy farms, no national surveys have been conducted on the prevalence of *Campylobacter* spp. in raw milk. Therefore, the objective of this study was to determine the overall prevalence of *Campylobacter* spp. in BTM and milk filters from US dairy operations.

2.3 Materials and Methods

2.3.1 Samples.

Samples were collected as part of the NAHMS Dairy 2014 study. Operations were chosen based upon data from the USDA's National Agricultural Statistics Service, which selected operations based on herd size from each of the 17 top dairy states (California, Idaho, Indiana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New Mexico, New York, Ohio, Pennsylvania, Texas, Vermont, Virginia, Washington, and Wisconsin) (Table 1). These states represented 80% of dairy herds and 80% of dairy cows in the US. The survey design was a stratified random sample with unequal selection probabilities in order to ensure the inclusion of large dairy operations.

Those producers (n=3500) reporting one or more milk cows in their inventory on 1 January 2014 were included in Phase I of the NAHMS Dairy 2014 study. In Phase I, National Agricultural Statistics Service enumerators administered a general management questionnaire to participating producers (n=1261). In Phase II, federal and state veterinary medicals officers or animal health technicians administered a veterinary questionnaire to operations (n=265) with 30 or more milk cows on 1 January 2014 that had participated in Phase I and agreed to continue participating in the study. From March 2014 to June 2014, trained staff aseptically collected BTM (50-100 mL) and milk filters from participating operations (n=234). The samples were shipped overnight on ice to the USDA, Agricultural Research Service in Beltsville, MD. Collected samples represented one milking cycle of the whole milking herd.

2.3.2 Bacteriological methods.

For enrichment of *Campylobacter* spp. from BTM, multiple tubes of milk from an operation were first combined and mixed, then 25 mL was centrifuged at 20,000 × *g* for 35 min. The supernatant was discarded, and the pellet was resuspended in 45 mL Bolton Broth (Oxoid, England) with 5% Laked Horse Blood (Lampire, Pipersville, PA) and a *Campylobacter* selective supplement that contained cefoperazone, cycloheximide, trimethoprim, and vancomycin (Dalynn, Canada). For enrichment of *Campylobacter* spp. from milk filters, the filters were cut into small pieces, placed in filtered stomacher bags, and 1% buffered peptone water (1:1, wt/wt) was added. The bags were pummeled in an automatic bag mixer for 2 min, repositioned and then pummeled for an additional 2 min. Five mL of filtrate was added to 40 mL Bolton Broth with 5% Laked Horse Blood and a selective supplement that contained cefoperazone, cycloheximide, trimethoprim, and vancomycin.

For all samples, enrichment tubes were incubated at 37°C for 48 h in a 10% CO_2 incubator with loosened caps. After incubation, 2 mL of enrichment broth were centrifuged (12,000 × *g*). The supernatants were discarded, and the bacterial pellets were suspended in 0.5 mL of 1X freezer medium (Schleif and Wensink 1981) and stored at -80°C. Additionally, 1.5 mL of enrichment broth was centrifuged (12,000 × *g*), and nucleic acids were extracted from the bacterial pellets using 200 μL of a commercially prepared extraction medium (mericon DNA Bacteria Kit Fast Lysis Buffer, Qiagen, Germany).

After incubation, 30 μL of enrichment broth was also streaked onto *Campy* Blood-Free Selective Medium (Neogen, Lansing, MI) which was supplemented with cefoperazone (Neogen, Lansing, MI) and incubated at 42°C for 48 h in a microaerophilic environment (10% CO_2, 5% O_2, 85% N_2) using an Anoxomat canister system (Mart Microbiology, The Netherlands). Two to five suspected *Campylobacter* spp. colonies were transferred from each plate to a panel of plates that included Campy Blood-Free Selective Medium, Columbia Agar with 5% Sheep Blood (BD, Sparks, MD), Brilliance CampyCount (Remel, Lenexa, KS), *C. jejuni/C. coli* Chromogenic Plating Medium (R&F Laboratories, Downers Grove, IL), and Mueller Hinton (Neogen, Lansing, MI) agar plates. DNA was extracted from one unique isolate from each sample using an InstaGene Matrix suspension (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Biomass from isolates was preserved in 500 μL of a freezer medium consisting of 80% Bolton Broth, 15% glycerol and 5% Laked Horse Blood and stored at -80°C.

2.3.3 Real-time PCR.

The DNA preparations that were extracted from enrichments were analyzed by real-time PCR for the presence of *C. jejuni, C. coli and C. lari* using a commercial kit (mericon *Campylobacter* triple kit, Qiagen, Germany) following the manufacturer's directions. Presumptive *Campylobacter* spp. isolates were also confirmed as such by a commercial real-time PCR kit. Reactions were run on a Stratagene Mx3005P qPCR system (Stratagene, La Jolla, CA) following the kit

manufacturer's cycling protocol. Samples with a cycle threshold of less than 40 cycles were considered positive.

2.3.4 Species identification.

Campylobacter species were determined by multiplex PCR of the isolate DNA preparations as described by Wang et al. (2002) with modifications. Each reaction mixture contained 240 μM deoxynucleoside triphosphate mixture (ThermoFisher, Lenexa, KS); 2.5 μL of 10X reaction buffer II; 4 mM $MgCl_2$; 1.5 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA); 0.6 μM *C. jejuni* and *C. lari* primers; 1 μM *C. coli* and *C. fetus* primers; 2 μM *C. upsaliensis* primers; 0.2 μM 23S rRNA primers; 1.0 μL of chromosomal DNA; and molecular biology grade water to a final volume of 26 μL. The reaction conditions were an initial incubation at 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 7 min. PCR reactions were run on a 1.5% agarose gel in 1X Tris-borate-EDTA buffer (Invitrogen, Grand Island, NY). *C. jejuni* NCTC 81116, *C. coli* ATCC 33559, *C. lari* 43675, *C. fetus* subsp. *fetus* ATCC 33246, and *C. upsaliensis* ATCC 43954 were utilized as positive controls.

2.3.5 Pulsed-field gel electrophoresis analysis.

Molecular typing by pulsed-field gel electrophoresis (PFGE) was performed following the standardized PulseNet *C. jejuni* protocol (Ribot et al., 2001) with a few modifications. Cultures were struck onto Columbia Blood Agar plates, and incubated at 42° for 48 h, and biomass from these plates was used for agarose plug preparation. The DNA in the plug slices was digested overnight with 40 U *Sma*I. *Xba*I-digested *S. enterica* serotype Braenderup H9812 was the size

strain. Thiourea (50 μM) was added to both the gel and the electrophoresis buffer. Gels, composed of 1% SeaKem Gold agarose in Tris-borate-EDTA, were run on CHEF-DR II or CHEF-DR III systems for 18.5 h. The gels were stained with 1μg/mL ethidium bromide and destained with 4 washings. Images of the restriction digest patterns were obtained using a ChemiDoc XRS gel documentation system (Bio-Rad, Hercules, CA). Bands were manually assigned and analyzed using BioNumerics software (Applied Maths, Austin, TX). Dendrograms were derived using the unweighted pair group method with arithmetic average cluster analysis with a band position tolerance of 1.0% and optimization of 0.5%.

2.3.6 Antimicrobial susceptibility testing.

Resistance to a panel of 9 commonly used antimicrobials (azithromycin, ciprofloxacin, erythromycin, gentamicin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin) was assessed using an automated microdilution procedure (Sensititre, ThermoFisher, Lenexa, KS). Antimicrobial minimum inhibitory concentrations (MICs) were interpreted based upon the epidemiological cut-off values (ECOFFs) used by the National Antimicrobial Resistance Monitoring System (NARMS) (Table 2). *C. jejuni* ATCC 33560 was used as a susceptible quality control strain.

2.4 Results

A total of 234 BTM samples and 231 milk filter samples were received between March and June 2014 (Table 1). Paired samples of milk and filters were received from 231 dairy operations and 3 operations submitted only milk samples.

Field personnel were instructed to collect milk samples and all associated milk filters from a single milking for the herd. The number of filters collected from individual herds ranged from 1 to 20 filters. When necessary, filters from the same operation were divided into multiple enrichments; however, results from multiple filter samples from the same operation were combined for analysis. Overall, samples were received from 234 operations in 17 states.

When DNA extracts from enrichment biomass was tested for the presence of thermophilic *Campylobacter* spp. (*C. jejuni, C. coli, C. lari*), samples with a real-time PCR cycle threshold of less than 40 cycles were considered positive. The cycle thresholds of positive samples ranged from 14.3 to 39.5. Based on real-time PCR analysis, 27 milk samples and 69 filter samples were positive for the presence of *C. jejuni, C. coli* or *C. lari*, representing 11.5% and 29.5% of tested operations, respectively (Table 3). Cycle thresholds greater than 32 were obtained from 41 of the 96 PCR-positive samples. Overall, enrichments from 80 operations (34.2%) were positive for thermophilic *Campylobacter* species. Sixteen operations (6.8%) tested positive for *Campylobacter* spp. in both the BTM and filter samples. *Campylobacter* was detected in the filter sample alone for 53 operations (22.6%) while it was detected in the BTM sample alone for 9 operations (3.8%). *Campylobacter* was also detected in 2 BTM samples (0.9%) that were submitted without accompanying filter samples.

Campylobacter spp. were isolated from the filter samples of 33 operations and from the BTM samples of 14 operations, representing a total of 43 operations (18.4%). Isolates were obtained from both the BTM and filter samples of 4

operations (1.7%). *Campylobacter* spp. were isolated from only the filter samples from 29 operations (12.4%) and from only the BTM samples from 9 operations (3.8%). Isolates were also obtained from one BTM sample (0.4% of operations) that did not have an accompanying filter sample.

When 49 *Campylobacter* isolates were analyzed using multiplex PCR, 45 isolates (91.8%) were identified as *C. jejuni*, 3 isolates were *C. lari* and 1 isolate was *C. coli*. Of these 49 isolates, one *C. jejuni* isolate and one *C. lari* isolate were obtained from a single filter sample (Pair 23). Seven isolates could not be resuscitated from preservation and one *C. jejuni* isolate repeatedly failed to grow on the microdilution plate used for susceptibility testing. The remaining 41 *Campylobacter* spp. isolates were tested for resistance to 9 common antibiotics utilizing an automated microdilution procedure (Table 4). Both *C. lari* isolates were resistant to ciprofloxacin and nalidixic acid while the *C. coli* isolate was susceptible to all 9 antibiotics. Of the 38 *C. jejuni* isolates tested, 26 isolates (68.4%) were resistant to tetracycline. Five (13.2%) of these tetracycline-resistant *C. jejuni* isolates were also resistant to both ciprofloxacin and nalidixic acid. Twelve *C. jejuni* isolates (31.6%) were susceptible to each of the 9 antibiotics.

In total, 42 *Campylobacter* isolates were analyzed by PFGE. This included the one C. *jejuni* isolate for which antimicrobial susceptibility could not be determined. The PFGE banding patterns of the *Sma*I digests of *C. jejuni* isolates were composed of 6 to 10 fragments (Figure 1). The *C. coli* isolate was composed of 7 fragments while the 2 *C. lari* isolates were composed of 4 and 7 fragments. When the banding patterns of all isolates were compared, the 2 *C. lari* and one *C.*

coli isolate were distinct, sharing less than 35% similarity with the *C. jejuni* isolates. While the 39 *C. jejuni* isolates clustered separately from *C. lari* and *C. coli*, significant diversity was observed within the species, and this diversity extended across geographical regions. *C. jejuni* isolates grouped into 4 clades, with each sharing at least 50% similarity between isolates within each clade. Eight isolate pairs and one group of 3 isolates were indistinguishable (100% similarity). Of these 9 groups, 4 represented isolates from the same state whereas 5 were from differing, and generally non-neighboring, states. Three indistinguishable pairs represented isolates from BTM and milk filter pairs from the same operations. Antimicrobial resistant phenotypes were dispersed across clades. Of the 9 indistinguishable groups, 7 groups displayed identical antimicrobial resistance or susceptibility profiles.

When the PFGE patterns of the *Campylobacter* isolates were compared with the CDC's PulseNet database, 35 *C. jejuni* isolates and 1 *C. coli* isolate matched strains isolated from human outbreaks in 2015. The pulsetypes of seven these isolates matched *C. jejuni* outbreak pulsetypes of isolates from raw milk. Four isolates from 4 different states matched a highly-virulent *C. jejuni* strain noted for its role in ruminant abortions and resistance to tetracycline. Of the 36 *Campylobacter* isolates, 26 matched pulsetypes in the database that were obtained from poultry sources, particularly chicken breasts.

2.5 Discussion

Campylobacter spp. are a leading cause of human foodborne illness. While sporadic infections are typically associated with poultry, outbreaks have been attributed to milk and dairy products, with the majority of outbreaks attributed to unpasteurized or raw milk (Taylor et al., 2013; Mungai, 2015). Recurrent outbreaks of campylobacteriosis from raw milk sold by one Pennsylvania farm occurred in both 2012 and 2013, leading to illness in multiple states (CDC, 2013; Longenberger et al., 2013). Although far fewer, *Campylobacter* outbreaks have also been attributed to pasteurized milk, potentially due to post-pasteurization contamination (Taylor et al., 2013). Previous national surveys have determined that *Campylobacter* spp. can be detected on nearly all dairy farms, and multi-state surveys have also isolated *Campylobacter* spp. from BTM. (NAHMS, 2002; NAHMS, 2007; Halbert et al., 2006)

Campylobacter spp. were identified in about one-third of the BTM and filter samples collected from US dairy operations as part of the NAHMS 2014 Dairy study. Thermophilic *Campylobacter* spp. *(C. jejuni, C. lari, C. coli*) were detected in samples collected from 34.2% of operations from 17 top dairy states in the US. This survey involved a single point-in-time sampling and therefore may underestimate the contamination. Previous research indicates that *Campylobacter* spp. may be shed seasonally in dairy animals, with peaks in the spring in Wisconsin and peaks in spring and fall in the United Kingdom (Stanley et al.,

1998; Sato et al., 2004). Therefore, the prevalence of *Campylobacter* spp. in BTM could vary along with seasonal fluctuations in shedding.

Campylobacter spp. isolates were obtained from approximately half of the samples that were identified as positive via PCR (18.4% vs 34.2%). *Campylobacter* spp. are challenging to culture in the laboratory and require a microaerophilic environment as well as supplementation with antibiotics to inhibit competition. Although *Campylobacter* spp. were not isolated from all samples in which detected, a prevalence of 18.4% via culture methods represents an increase from previous multi-state surveys, which isolated *Campylobacter* spp. from between 0.9% and 12.3% of BTM samples. (Doyle and Roman, 1982; Lovett et al., 1983; McManus and Lanier, 1987; Rohrbach et al., 1992).

C*ampylobacter* spp. were detected in the filter samples but not in the BTM samples from 53 operations (22.6%). Previous work with *Campylobacter* spp. and other dairy-associated pathogens has illustrated the value of milk filter sampling as a more sensitive method of detecting of bacterial pathogens than BTM sampling alone (Giacometti et al., 2012; Latorre et al., 2011; Leone et al., 2010; Serraino et al., 2013; Van Kessel et al., 2008; Van Kessel et al., 2011). A multi-state survey of 128 organic and conventional farms in the Michigan, Minnesota, New York, and Wisconsin obtained 11 isolates from milk filters, but only 3 isolates from BTM samples (Halbert et al., 2006). In comparison, surveys of BTM and filters in Italy, where raw milk is legally sold, detected *Campylobacter* spp. in 22.2% to 63% of milk filter samples (Giacometti et al., 2012; Serraino et al.,

2013; Leone et al., 2010) and 12% to 68% of BTM samples (Leone et al., 2010; Bianchini et al., 2014).

Although most frequently identified in the milk filters, *Campylobacter* spp. were also isolated from the BTM from 9 operations while the filter samples from the same operation were negative. Detection in the BTM but not the filter may be due to the sensitivity of *Campylobacter* spp. to environmental conditions, such as drying or freezing (Cox et al., 2001). *Campylobacter* spp. require an elevated water activity level (0.987 to 0.997) and cannot withstand temperature fluctuations or increased oxygen concentrations (Silva et al., 2011). Filter samples were shipped overnight in reclosable bags; however, the condition of the filters upon arrival varied, potentially impacting the ability to recover *Campylobacter* spp. from the sample. Although producers and field staff received instructions on proper collection technique, some filters appeared dry or were shipped in unsealed bags. Other filter samples appeared to have been rinsed with water. Therefore, the sensitivity of the organism combined with the inconsistency in sample condition may have reduced the ability to obtain *Campylobacter* spp. isolates.

C. jejuni is the most frequent *Campylobacter* species associated with dairy animals and their environment and with human illness (CDC, 2014) and was the predominant species isolated in this study. Of the 49 isolates obtained from BTM and milk filter samples, 45 were *C. jejuni.* One *C. jejuni* was isolated from a milk filter from which *C. lari* was also isolated. When the susceptibility of these isolates to a panel of common antimicrobials was tested, twelve *C. jejuni* isolates (31.6%) were susceptible to all 9 antimicrobials; however, 68.4% of *C. jejuni*

isolates were resistant to tetracycline (Table 4). Although BTM and milk filters were not tested for the presence of *Campylobacter* spp. in the previous NAHMS 2007 study, 62.5% of *C. jejuni* isolates from fecal samples in 2007 were also resistant to tetracycline. Tetracycline and its derivatives are commonly used on dairy operations in milk replacers and foot baths; therefore, resistance in the dairy environment is not unexpected (NAHMS, 2007; Zwald et al., 2004). Five of the tetracycline-resistant isolates were also resistant to ciprofloxacin and nalidixic acid. Campylobacteriosis in humans, most frequently due to *C. jejuni*, is usually self-limiting; however, when antimicrobial intervention is deemed necessary, erythromycin is the drug of choice. Ciprofloxacin may also be used as an alternative; however, the most recent national monitoring survey of antimicrobial resistance in the US identified resistance to ciprofloxacin in 22% of human *C. jejuni* isolates and 8.5% of dairy fecal isolates (NARMS, 2013).

C. coli is the second most common cause of human infection. One *C. coli* isolate from a milk filter was susceptible to all tested antimicrobials. While *C. lari* causes human illness infrequently, it is intrinsically resistant to quinolones (Skirrow and Benjamin, 1980; CDC, 2014). Resistance to the quinolones, ciprofloxacin and nalidixic acid, was observed in the 2 *C. lari* isolated from milk filter samples.

When the PFGE *Sma*I restriction digest patterns were compared, the diversity within *C. jejuni* isolates and their distinction from *C. lari* and *C. coli* were observed. In two large surveys of fecal samples from cows on US dairy farms, Harvey (2005) and Sanad (2011) similarly noted diversity in the digest

patterns of *Campylobacter* spp. isolated from multiple states. This diversity has been attributed to transmission of *Campylobacter* to dairy animals from migratory birds, wildlife, livestock, or humans. Additionally, *Campylobacter* spp. are naturally competent and also rapidly evolve, with mutations within the population occurring within the course of a week (Young et al., 2007; Wilson et al., 2009). When comparing the restriction digest patterns of isolates from the BTM and filters, several *C. jejuni* isolate groups were indistinguishable. Five of these indistinguishable groups were obtained from operations in different states while three indistinguishable groups of isolates represented BTM and milk filter pairs from the same operation. A fourth BTM-milk filter isolate pair (Pair 196) yielded two distinct PFGE patterns, illustrating the diversity of *C. jejuni* even at the farm level. This farm-level diversity could be due to the infection and shedding of different strains by multiple cows or, alternatively, the shedding of multiple strains by the same cow. Nielson (2002) similarly observed that individual animals could shed multiple serotypes.

When restriction digest patterns were compared with antimicrobial resistance phenotypes, no consistent association was observed. Harvey (2005) similarly observed that antimicrobial resistance patterns differed within genotypes of isolates from three geographic regions (northeast, southwest, west) despite similar genotypic patterns throughout these regions. In this study, resistance to tetracycline was observed throughout the four *C. jejuni* clades and across geographic regions (Figure 1). In each of 2 pairs of indistinguishable *C. jejuni* isolates, one isolate was resistant to tetracycline while the second was

pansusceptible. Resistance to ciprofloxacin and nalidixic acid was observed in isolates from two separate *C. jejuni* clades. Therefore, *C. jejuni* isolates are geographically dispersed and have varying degrees of resistance between indistinguishable isolates and across both clades and geographic regions.

The CDC collects PFGE patterns of pathogenic isolates from humans and other sources in the PulseNet database in order to detect foodborne outbreaks. When the PFGE patterns of the *Campylobacter* spp. isolates were compared with the PulseNet database, 35 of 39 *C. jejuni* isolates matched isolates from human outbreaks from 2015, of which 7 were isolated from raw milk. The PFGE pattern of 4 isolates matched a highly-virulent *C. jejuni* strain, known as clone SA (Sahin et al., 2012). Clone SA is associated with abortions in ruminants; however, the isolates from the 2015 PulseNet database were originally isolated from chickens. An additional 22 isolates matched isolates in the database that were obtained from poultry sources, such as chicken breasts, indicating that *Campylobacter* is not host-specific.

Campylobacter spp. are not only present on most dairy farms in the US, but can also contaminate BTM. *Campylobacter* spp. were detected in the BTM and milk filters from one-third of US operations tested as part of the NAHMS Dairy 2014 study. Additionally, 68.4% of *Campylobacter* spp. isolates were resistant to at least one antimicrobial. Although *Campylobacter* spp. can be killed through proper pasteurization, there is a risk of foodborne illness for a segment of the public that consumes unpasteurized, raw milk.

Chapter 3: Conclusion

Campylobacter spp. are the primary cause of foodborne outbreaks attributed to raw milk and can be isolated from most US dairy environments. To date, no national study has determined the prevalence of *Campylobacter* spp. in unpasteurized, raw BTM on US dairy farms. The results of this study suggest that *Campylobacter* spp. are a common contaminant of BTM on US dairies and confirm the results of previous multi-state surveys of BTM. Although proper pasteurization can eliminate *Campylobacter* spp., there is clearly a risk of foodborne illness to consumers of unpasteurized, raw milk. Further analysis of data collected as part of this study may also compare *Campylobacter* prevalence to farm location, size and management practices in order to identify where improvements can be implemented to limit *Campylobacter* contamination in the BTM. A risk assessment comparing both the hazards and benefits of unpasteurized and pasteurized milk would also be valuable. Together, the prevalence, antimicrobial resistance and molecular typing results from this study will contribute to the evidence of *Campylobacter* spp. prevalence on US dairy farms. These results provide support to the positions of many major organizations, such as the National Mastitis Council and the American Academy of Pediatrics, that advise against the consumption of unpasteurized, raw milk.

Tables

Table 1. State of origin and summary of bulk tank milk samples and milk filter samples received from March to June 2014

State	No. of operations	Sample counts (no.)			
		Milk	Filter	Pairs[1]	Milk, no filters[2]
CA	22	22	22	22	0
CO	5	5	5	5	0
ID	6	6	6	6	0
IN	3	3	3	3	0
IA	12	12	12	12	0
KY	1	1	1	1	0
MI	18	18	17	17	1
MN	24	24	24	24	0
MO	2	2	2	2	0
NY	33	33	33	33	0
OH	10	10	10	10	0
PA	19	19	19	19	0
TX	1	1	1	1	0
VT	1	1	1	1	0
VA	8	8	7	7	1
WA	11	11	11	11	0
WI	58	58	57	57	1
Total	234	234	231	231	3

[1] Number of operations from which there was both a bulk tank milk sample and a filter sample.

[2] Number of operations from which milk samples were submitted but there was no corresponding filter sample from the same operation.

Table 2. Epidemiological cut-off values used to determine resistance[1,2]

Antimicrobial	*C. jejuni/ C. lari*[3]	*C. coli*
Azithromycin	≥ 0.5	≥ 1
Ciprofloxacin	≥ 1	≥ 1
Erythromycin	≥ 8	≥ 16
Tetracycline	≥ 2	≥ 4
Florfenicol	≥ 8	≥ 8
Nalidixic Acid	≥ 32	≥ 32
Telithromycin	≥ 8	≥ 8
Clindamycin	≥ 1	≥ 2
Gentamicin	≥ 4	≥ 4

[1]Based on The European Committee on Antimicrobial Susceptibility Testing
[2]Minimum Inhibitory Concentrations (MICs)/ECOFFs, μg/mL
[3]No specific ECOFFs for *C. lari*

Table 3. Number and percent of US dairy farms that tested positive for *Campylobacter* spp. by PCR and culture in bulk tank milk and milk filters[1]

Sample type and result	PCR		Culture	
	No. of operations	%	No. of operations	%
Positive milk	27	11.5	14	6.0
Positive filter	69	29.5	33	14.1
Positive milk and positive filter	16	6.8	4	1.7
Negative milk and positive filter	53	22.6	29	12.4
Positive milk and negative filter	9	3.8	9	3.8
Positive milk and no filter sample	2	0.9	1	0.4
Operations with any positive sample	80	34.2	43	18.4

Table 4. Resistance and resistance profiles of *Campylobacter* spp. isolates (n=41) from bulk tank milk and milk filters from US dairies.

			No. of isolates	% of isolates
C. jejuni	Resistance	Tetracycline	26	68.4
		Ciprofloxacin	5	13.2
		Nalidixic Acid	5	13.2
		Pansusceptible[2]	12	31.6
	Resistance Profile	Tetracycline	21	55.2
		Tetracycline *and* Ciprofloxacin *and* Nalidixic Acid	5	13.2
		Pansusceptible[1]	12	31.6
C. lari	Resistance	Ciprofloxacin	2	100.0
		Nalidixic Acid	2	100.0
	Resistance Profile	Ciprofloxacin *and* Nalidixic Acid	2	100.0
C. coli		Pansusceptible[1]	1	100.0

[1]Susceptible to azithromycin, ciprofloxacin, erythromycin, tetracycline, florfenicol, nalidixic acid, telithromycin, clindamycin, and gentamicin.

Figures

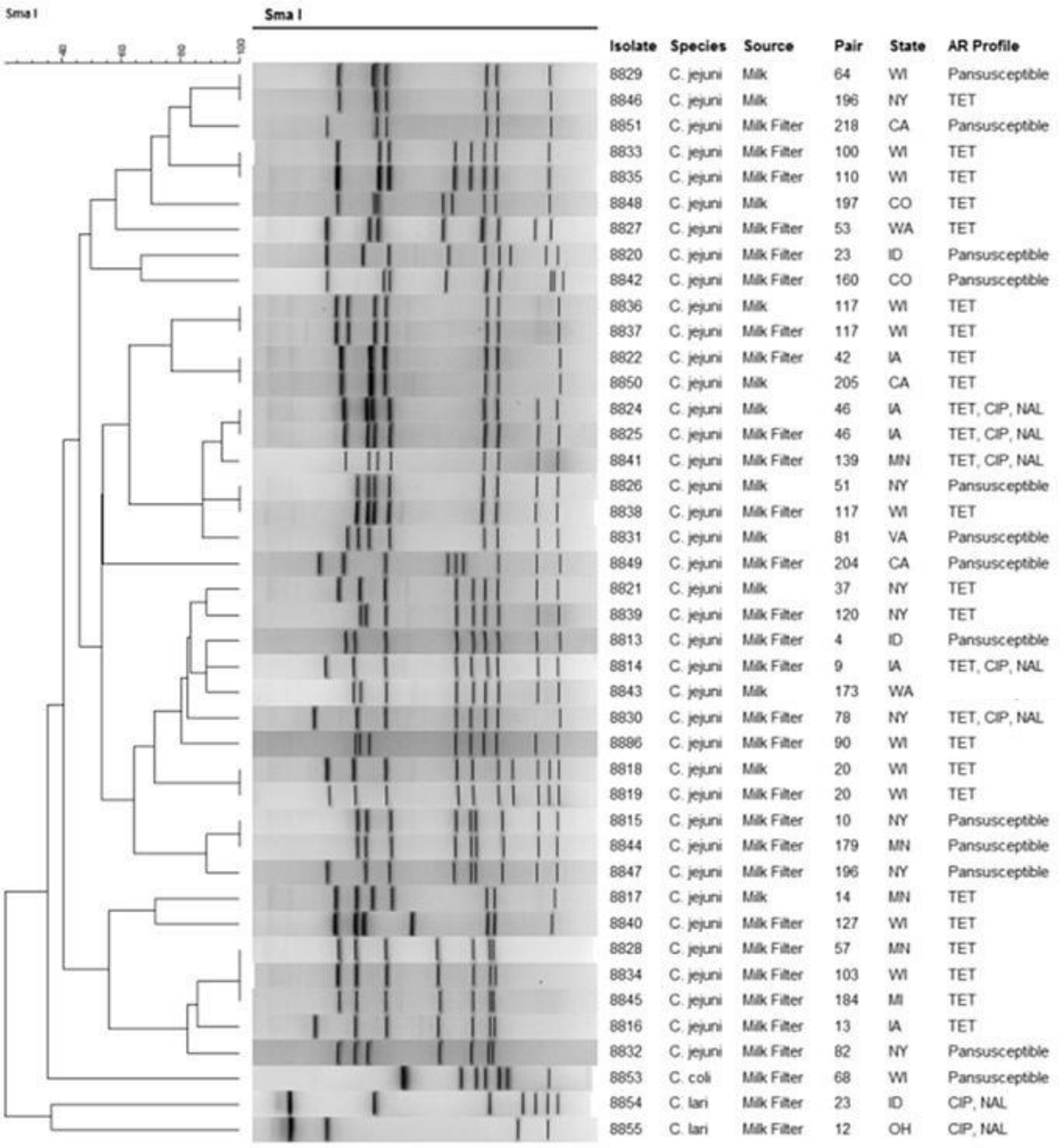

Figure 1. Dendrogram of *Sma*I-digested pulsed-field gel electrophoresis patterns. Abbreviations: TET (tetracycline), CIP (ciprofloxacin), and NAL (nalidixic acid).

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