Prevalence, antimicrobial resistance, and molecular characterization of *Campylobacter* spp. in bulk tank milk and milk filters from US dairies

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ABSTRACT

Campylobacter spp. are frequently isolated from dairy cows as commensal organisms. Sporadic *Campylobacter* infections in humans in the United States are generally attributed to poultry, but outbreaks are also commonly associated with dairy products, particularly unpasteurized or raw milk. Bulk tank milk samples and milk filters from US dairy operations were collected during the National Animal Health Monitoring System Dairy 2014 study and analyzed using real-time PCR and traditional culture techniques for the presence of thermophilic *Campylobacter* species. The weighted prevalence of operations from which we detected *Campylobacter* spp. in either bulk tank milk or milk filters was 24.9%. We detected *Campylobacter* spp. in a higher percentage of operations with 100–499 cows (42.8%) and 500 or more cows (47.5%) than in operations with 30–99 cows (6.5%). *Campylobacter* spp. were also more frequently detected in operations in the west than the east (45.9 and 22.6%, respectively). We isolated *Campylobacter* spp. from approximately half of PCR-positive samples, representing 12.5% (weighted prevalence) of operations. The majority (91.8%) of isolates were *C. jejuni*, but *C. lari* and *C. coli* were also isolated. We detected resistance to tetracycline in 68.4% of *C. jejuni* isolates, and resistance to ciprofloxacin and nalidixic acid in 13.2% of *C. jejuni* isolates. Based on pulsed-field gel electrophoresis, we found that dairy-associated *C. jejuni* were genotypically diverse, although clonal strains were isolated from different geographic regions. These results suggest that bulk tank milk can be contaminated with pathogenic *Campylobacter* spp., and that the consumption of unpasteurized or raw milk presents a potential human health risk.

Key words: *Campylobacter*, bulk tank milk, milk filter

INTRODUCTION

Campylobacter spp. are microaerophilic, spiral-shaped, gram-negative bacteria that are a leading cause of bacterial diarrhea in humans in the United States, accounting for an estimated 1.3 million cases and 120 deaths annually (CDC, 2016). Campylobacteriosis is usually self-limiting, with symptoms such as mild diarrhea and cramping; however, severe cases may require antimicrobial treatment. Post-infection sequelae such as Guillain-Barré syndrome may cause lifelong health problems. According to preliminary 2015 data published by the Centers for Disease Control and Prevention (CDC), *Campylobacter* spp. infections in humans increased by 9% from 2006 to 2015 (CDC, 2016). The most common cause of campylobacteriosis in humans is *Campylobacter jejuni*, but other thermophilic species such as *Campylobacter coli* and *Campylobacter lari* have also been known to cause disease (Taylor et al., 2013; CDC, 2016).

Although sporadic *Campylobacter* spp. infections are most commonly associated with poultry, outbreaks have also been attributed to the consumption of other foods, particularly unpasteurized or raw milk. Based on a review of CDC data from 1997 to 2008, 28% of foodborne *Campylobacter* outbreaks (defined as 2 or more affected people) were due to the consumption of contaminated raw milk products, compared with 11% due to the consumption of poultry (Taylor et al., 2013). Langer et al. (2012) and Mungai et al. (2015) analyzed milk-related outbreak data from 1993 to 2006 and 2007 to 2012, respectively, and found that the majority of outbreaks associated with unpasteurized milk were due to *Campylobacter* contamination. Furthermore, in a recent analysis of *Campylobacter* spp. isolated from human and animal sources, Tyson et al. (2016) discovered that isolates from human clinical samples more frequently matched isolates from dairy cattle (65.0%) than isolates from poultry sources (49.5%). Therefore,

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dairy cattle and dairy products, particularly unpasteurized milk, should be considered an important source of *Campylobacter* spp. infection.

Dairy cows are known reservoirs of *Campylobacter* spp., and several surveys have determined the prevalence of *Campylobacter* spp. on dairy farms. According to National Animal Health Monitoring System (NAHMS) studies in 1996, 2002, and 2007, *Campylobacter* spp., primarily *C. jejuni*, were detected in the feces of cows on 90–100% of US dairy operations (USDA-APHIS, 2011). The within-herd prevalence of most operations was greater than 10%. Dairy cows have been described as intermittent shedders; however, cows may also shed *C. jejuni* consistently or sporadically in high concentrations (Häkkinen and Hänninen, 2009; Rapp et al., 2012). *Campylobacter* spp. have also been detected in bulk tank milk (BTM; Halbert et al., 2006). Although *Campylobacter* spp. have occasionally been implicated in cases of mastitis (Morgan et al., 1985; Orr et al., 1995; Bianchini et al., 2014) and can be excreted in the milk of infected cows, this is an uncommon occurrence, and the presence of *Campylobacter* spp. in BTM is usually due to fecal contamination (Oliver et al., 2005). Therefore, even with good animal hygiene and strict milking protocols, some level of contamination in the bulk tank by asymptomatic animals is inevitable.

Pasteurization is a highly effective tool for managing BTM contamination; however, raw milk can be legally distributed in some manner in more than half of US states (AAP, 2014; NCSL, 2016). Although dairy animals are common carriers of *Campylobacter* spp. and fecal shedding can lead to BTM contamination, no national surveys have been conducted on the prevalence of *Campylobacter* spp. in BTM in the United States. Sampling milk filters in addition to BTM can also improve the ability to detect pathogens in BTM (Van Kessel et al., 2008; Latorre et al., 2011; Van Kessel et al., 2011). The objective of this study was to determine the prevalence of *Campylobacter* spp. in BTM and milk filters from US dairy operations from samples collected during the NAHMS Dairy 2014 study.

MATERIALS AND METHODS

To identify operations that would participate in the NAHMS Dairy 2014 study, a stratified random sample of dairy operations was selected from the USDA National Agricultural Statistics Service list frame. The survey design was a stratified random sample with unequal selection probabilities to ensure the inclusion of dairy operations with 500 or more cows and organic operations. Operations were chosen from each of 17 dairy states in 2 regions of the country (west: California, Colorado, Idaho, Texas, and Washington; east: In-

diana, Iowa, Kentucky, Michigan, Minnesota, Missouri, New York, Ohio, Pennsylvania, Vermont, Virginia, and Wisconsin). These states represent 80.5% of dairy herds and 81.3% of dairy cows in the United States. Operations were classified into 3 herd size categories based on the number of cows: small (30–99 cows), medium (100–499 cows), and large (500 or more cows).

Producers ($n = 3,500$) that reported 1 or more milk cows in their inventory on January 1, 2014, were selected for phase I of the NAHMS Dairy 2014 study. During this phase, National Agricultural Statistics Service enumerators administered a general management questionnaire to participating producers ($n = 1,191$). In phase II, federal and state veterinary medical officers or animal health technicians administered an additional questionnaire to operations with 30 or more milk cows on January 1, 2014, that had participated in phase I and agreed to continue participating in the study ($n = 265$).

Samples from BTM and milk filters were collected from March to July 2014. Trained staff aseptically collected BTM (50–150 mL) and milk filters from participating operations. Sample collectors were instructed to ensure that the BTM and milk filter samples represented at least 1 complete milking cycle of the herd. Thus, if the herd had more than 1 bulk tank, then each bulk tank was sampled, and all milk filters used during a milking cycle were collected. Samples were shipped overnight on ice to the USDA-Agricultural Research Service in Beltsville, Maryland.

For enrichment of *Campylobacter* spp. from BTM, multiple tubes of milk from an operation were first combined and mixed, and then a 25 mL aliquot was centrifuged at $20,000 \times g$ for 35 min at 8°C (Figure 1). The supernatant was discarded, and the pellet was resuspended in 45 mL of Bolton Broth (Oxoid, Basingstoke, UK) with 5% Laked Horse Blood (Lampire, Pipersville, PA) and a *Campylobacter* selective supplement that contained cefoperazone, cycloheximide, trimethoprim, and vancomycin (Dalynn, Calgary, AB, Canada). For enrichment of *Campylobacter* spp. from milk filters, the filters were cut into small pieces and placed in filtered stomacher bags, to which 1% buffered peptone water (1:1, wt/wt) was added, as previously described by Van Kessel et al. (2011). The bags were pummeled in an automatic bag mixer for 2 min, repositioned, and then pummeled for an additional 2 min. Then, 5 mL of filtrate was added to 40 mL of Bolton Broth with 5% Laked Horse Blood and *Campylobacter* selective supplement.

For all samples, enrichment tubes were incubated at 37°C for 48 h in a 10% CO₂ incubator with loosened caps. After incubation, 2 mL of enrichment broth were centrifuged ($12,000 \times g$). The supernatants were

discarded, and the bacterial pellets were suspended in 0.5 mL of 1× freezer medium (Schleif and Wensink, 1981) and stored at -80°C . Additionally, 1.5 mL of enrichment broth was centrifuged ($12,000 \times g$), 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, Hilden, Germany). The DNA preparations were analyzed by real-time PCR for the presence of *C. jejuni*, *C. coli*, or *C. lari* using a commercial kit (Mericon *Campylobacter* triple kit; Qiagen) following the manufacturer's instructions. Reactions were run on an Agilent Mx3005P qPCR system (Agilent Technologies, Santa Clara, CA) following the kit manufacturer's cycling protocol. Samples with a cycle threshold of fewer than 40 cycles were considered positive.

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) 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, Norwood, MA). Suspected *Campylobacter* colonies (2 to 5) were transferred from each plate to a panel of agar 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) agar plates. Then, DNA was extracted from 1 phenotypically unique isolate from each sample using an InstaGene Matrix suspension (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Presumptive *Campylobacter* spp. isolates were confirmed as such using a commercial real-time PCR kit (Mericon *Campylobacter* triple kit; Qiagen). All isolates were also preserved in 0.5 mL of a freezer medium consisting of 80% Bolton Broth, 15% glycerol, and 5% Laked Horse Blood, and stored at -80°C .

The species of confirmed *Campylobacter* isolates was determined by multiplex PCR as described by Wang et al. (2002) with modifications. Each reaction mixture (26 μL) contained 240 μM of a total deoxynucleoside triphosphate mixture (ThermoFisher, Lenexa, KS); 2.5 μL of 10× reaction buffer II; 4 mM MgCl_2 ; 1.5 U of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA); 0.6 μM each of forward and reverse *C. jejuni* and *C. lari* primers; 1 μM each *C. coli* and *Campylobacter fetus* primers; 2 μM *Campylobacter upsaliensis* primers; 0.2 μM 23S rRNA primers; 1.0 μL of InstaGene extract; and molecular biology-grade water. The reaction conditions were as follows: 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. The PCR products were separated on a 1.5% agarose gel in 1× Tris-borate-EDTA buffer (Invitrogen, Grand Island, NY) containing 0.5 $\mu\text{g/mL}$ ethidium bromide (Bio-Rad) and visualized under UV light. Positive controls were *C. jejuni* NCTC 81116, *C. coli* ATCC 33559, *C. lari* 43675, *C. fetus* ssp. *fetus* ATCC 33246, and *C. upsaliensis* ATCC 43954.

Resistance to a panel of 9 antimicrobials used by the National Antimicrobial Resistance Monitoring System (NARMS; azithromycin, ciprofloxacin, erythromycin, gentamicin, tetracycline, florfenicol, nalidixic acid, telithromycin, and clindamycin) was assessed using an automated microdilution procedure (Sensititre; ThermoFisher). Cultures were streaked onto Columbia Agar with 5% Sheep Blood (BD) and incubated at 42°C for 48 h; biomass from these plates was used for testing. We used *C. jejuni* ATCC 33560 as a susceptible quality control strain. Antimicrobial MIC were interpreted based on the epidemiological cutoff values used by the NARMS program (Table 1). The MIC that inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of isolates were also calculated (Schwarz, et al., 2010).

The *Campylobacter* spp. isolates were characterized by pulsed-field gel electrophoresis (PFGE) following the standardized PulseNet *C. jejuni* protocol (Ribot

Figure 1. Schematic of bulk tank milk and milk filter sample and isolate processing. PFGE = pulsed-field gel electrophoresis.

Table 1. Minimum inhibitory concentrations ($\mu\text{g/mL}$) used to determine resistance of *Campylobacter* spp.¹

Antimicrobial	<i>C. jejuni/C. lari</i> ²	<i>C. coli</i>
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

¹Based on epidemiological cutoff values established by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org).

²No specific MIC for *C. lari*.

et al., 2001) with a few modifications. Cultures were streaked onto Columbia Agar with 5% Sheep Blood (BD) and incubated at 42° for 48 h; biomass from these plates was used for agarose plug preparation. Plug slices were incubated overnight with 40 U *Sma*I (Roche Diagnostics, Mannheim, Germany). The size standard was *Xba*I-digested *Salmonella enterica* serotype Braenderup H9812. Thiourea (50 μM) was added to both the gel and the electrophoresis buffer. Gels composed of 1% SeaKem Gold agarose (Lonza, Allendale, NJ) in Tris-borate-EDTA (Sigma, St. Louis, MO) were run on Chef-DR II or Chef-DR III systems (Bio-Rad) for 18.5 h. The gels were stained with 1 $\mu\text{g/mL}$ ethidium bromide (Bio-Rad) and destained with 4 washings. Images of the restriction digest patterns were obtained using a

ChemIDoc XRS gel documentation system (Bio-Rad). Bands were assigned manually and analyzed using BioNumerics software (Applied Maths, Austin, TX). Dendrograms were derived using the unweighted pair group method with arithmetic average cluster analysis, a band position tolerance of 1.0%, and optimization of 0.5%.

The prevalence (%) of *Campylobacter* spp. at the operation level was statistically weighted to provide national prevalence estimates. The weighted prevalence (**wp**) was calculated using statistical software (SU-DAAN, release 11.0.1, 2013; Research Triangle Institute, Research Triangle Park, NC), which accounted for the operations' probability of selection and adjusted for non-response. Selection weights and non-response adjustments were based on state and herd size. Estimates of the percentage of positive samples were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

A total of 234 BTM and 231 milk filter samples were collected from 234 operations in 17 states between March and July 2014 (Table 2). Paired BTM and filter samples were received from 231 dairy operations and BTM only from 3 operations. The number of filters collected from individual operations ranged from 1 to 20. When necessary, filters from the same operation were processed in multiple enrichments (≤ 6 filters per en-

Table 2. State of origin and summary of bulk tank milk (BTM) samples and milk filter samples received from March to July 2014

State	No. of operations	Sample counts (no.)			
		BTM	Filter	Pairs ¹	BTM, no filters ²
California	22	22	22	22	0
Colorado	5	5	5	5	0
Idaho	6	6	6	6	0
Indiana	3	3	3	3	0
Iowa	12	12	12	12	0
Kentucky	1	1	1	1	0
Michigan	18	18	17	17	1
Minnesota	24	24	24	24	0
Missouri	2	2	2	2	0
New York	33	33	33	33	0
Ohio	10	10	10	10	0
Pennsylvania	19	19	19	19	0
Texas	1	1	1	1	0
Vermont	1	1	1	1	0
Virginia	8	8	7	7	1
Washington	11	11	11	11	0
Wisconsin	58	58	57	57	1
Total	234	234	231	231	3

¹Number of operations from which both a BTM sample and a filter sample were available.

²Number of operations from which BTM samples were submitted but no corresponding filter sample was available.

richment); however, results from multiple filter samples from the same operation were combined for analysis.

Based on real-time PCR analysis, the weighted prevalence of operations from which *Campylobacter* spp. were detected in either the BTM sample or the milk filters was 24.9% (Table 3). Of the 234 BTM and 231 milk filter samples, 27 BTM and 69 filter samples were positive for *C. jejuni*, *C. coli*, or *C. lari*. Real-time PCR cycle thresholds of positive samples ranged from 14.3 to 39.5. Overall, enrichments from 80 operations (24.9%, wp) were positive for *Campylobacter* species. Sixteen operations (5.3%, wp) tested positive for *Campylobacter* spp. in both the BTM and filter samples. *Campylobacter* spp. were detected in the filter sample but not the BTM for 53 operations (15.1%, wp), and in the BTM but not the filter for 9 operations (4.3%, wp). *Campylobacter* spp. were detected in 2 milk samples (0.2%, wp) submitted without accompanying filter samples. *Campylobacter* spp. were detected in BTM or milk filters in a higher percentage of operations with 100–499 cows and 500 or more cows (42.8 and 47.5%, respectively) than in operations with 30–99 cows (6.5%, $P < 0.0001$). *Campylobacter* spp. were also more frequently detected in operations in the west than in the east (45.9 and 22.6%, respectively; $P = 0.021$).

Campylobacter spp. were isolated via traditional culture methods from the filter samples of 33 operations and from the BTM samples of 14 operations, representing a total of 43 operations (12.5%, wp). Isolates were obtained from both the BTM and filter samples of 4 operations (1.2%, wp). *Campylobacter* spp. were isolated from only the filter samples from 29 operations (9.4%, wp) and from only the BTM samples from 9 operations (1.8%, wp). Isolates were also obtained from 1 BTM sample (0.1%, wp) that did not have an accompanying filter sample.

When phenotypically unique isolates ($n = 49$) from BTM and milk filters from 43 operations were analyzed using multiplex PCR, 45 (91.8%) were identified as *C. jejuni*, 3 were *C. lari*, and 1 was *C. coli*. One milk filter sample yielded both a *C. jejuni* isolate and a *C. lari*

isolate. In preparation for antimicrobial susceptibility testing, 7 of these isolates could not be resuscitated from preservation, and 1 isolate repeatedly failed to grow on the microdilution plate used for susceptibility testing. Therefore, 41 *Campylobacter* spp. isolates (38 *C. jejuni*, 2 *C. lari*, 1 *C. coli*) were tested for resistance to 9 common antimicrobials (Tables 4 and 5). Of the 38 *C. jejuni* isolates tested, 26 (68.4%) were resistant to tetracycline, and 5 (13.2%) of those were also resistant to both ciprofloxacin and nalidixic acid. Twelve *C. jejuni* isolates (31.6%) were susceptible to all of the 9 antimicrobials. Both *C. lari* isolates were resistant to ciprofloxacin and nalidixic acid, but the *C. coli* isolate was susceptible to all 9 antimicrobials.

Of the 49 *Campylobacter* isolates from BTM and milk filters, 42 were analyzed by PFGE (Figure 2). This included 1 *C. jejuni* isolate for which antimicrobial susceptibility could not be determined due to a failure to grow on the microdilution plate, but excluded 7 isolates that could not be resuscitated from preservation. When the banding patterns of all isolates were compared, the 2 *C. lari* isolates and 1 *C. coli* isolate were distinct, sharing less than 40% similarity with the *C. jejuni* isolates. The 39 *C. jejuni* isolates clustered separately from *C. lari* and *C. coli*, but we observed significant diversity within the species, and this diversity extended across geographical regions. The *C. jejuni* isolates grouped into 4 clades, each sharing at least 50% similarity between isolates within the clade. Eight groups of 2 isolates and 1 group of 3 isolates were indistinguishable (100% similarity). Of these 9 groups, 4 represented isolates from the same state, and 5 were from differing (and generally non-neighboring) states. Three indistinguishable isolate groups represented isolates from BTM and milk filter pairs from the same operations (pairs 20, 46, 117). Because filters were processed as multiple enrichments when necessary, 1 operation (pair 117) yielded 2 isolates from the milk filter sample as the result of separate enrichments. Although 1 isolate from 1 filter enrichment was indistinguishable (100% similarity) from the isolate from BTM, the isolate from

Table 3. Weighted percent of US dairy operations that were positive for *Campylobacter* (*C. jejuni*, *C. coli*, or *C. lari*) by real-time PCR and culture in bulk tank milk ($n = 234$ operations) and milk filters ($n = 231$ operations)

Sample type and result	PCR			Culture		
	Number	Weighted %	SE	Number	Weighted %	SE
Positive milk	27	9.8	2.3	14	3.0	1.1
Positive filter	69	20.5	3.0	33	10.6	2.3
Positive milk and positive filter	16	5.3	1.8	4	1.2	0.9
Negative milk and positive filter	53	15.1	2.5	29	9.4	2.1
Positive milk and negative filter	9	4.3	1.7	9	1.8	0.7
Positive milk and no filter sample	2	0.2	0.1	1	0.1	0.1
Any positive sample	80	24.9	3.3	43	12.5	2.4

Table 4. Resistance and resistance profiles of *Campylobacter jejuni* isolates (n = 38) from bulk tank milk and milk filters from US dairies

Resistance	MIC range (μg/mL)	MIC ₅₀ ¹ (μg/mL)	MIC ₉₀ ¹ (μg/mL)	No. of resistant isolates	% of isolates
Azithromycin	0.03–0.12	0.06	0.06	0	0.0
Ciprofloxacin (CIP)	0.03–16	0.06	8	5	13.2
Clindamycin	≤0.03–0.25	0.12	0.25	0	0.0
Erythromycin	0.06–1	0.25	0.5	0	0.0
Florfenicol	0.25–1	1	1	0	0.0
Gentamicin	0.25–1	0.5	1	0	0.0
Nalidixic acid (NAL)	≤4–>64	≤4	>64	5	13.2
Telithromycin	0.25–4	0.5	1	0	0.0
Tetracycline (TET)	≤0.06–>64	>64	>64	26	68.4
Susceptible ²				12	31.6
Resistance profile					
TET				21	55.2
CIP-NAL-TET				5	13.2

¹MIC₅₀ = MIC that inhibits 50% of the isolates; MIC₉₀ = MIC that inhibits 90% of the isolates.

²Susceptible to all 9 antimicrobials.

a second filter enrichment was distinct (<65% similarity). A second BTM–milk filter pair from a single operation (pair 196) also yielded 2 unique isolates (<40% similarity). Antimicrobial-resistant phenotypes were dispersed across clades. Of the 9 indistinguishable groups, 7 displayed identical antimicrobial resistance or susceptibility profiles.

DISCUSSION

Campylobacter spp. are a leading cause of foodborne gastrointestinal illness in the United States, accounting for an estimated 13.3 infections per 100,000 people in 2014 (CDC, 2016). Most illnesses are associated with poultry, but *Campylobacter* outbreaks are also often attributed to the consumption of raw milk (Taylor et al., 2013). The presence of *Campylobacter* in raw milk is primarily due to fecal contamination. Dairy cows are known reservoirs of *Campylobacter*; national surveys have consistently identified *Campylobacter* spp. on US dairy farms, particularly in fecal samples (USDA-APHIS, 2011). Regional and multi-state surveys have also frequently identified *Campylobacter* spp. in BTM on US dairy farms (Doyle and Roman, 1982; Lovett et

al., 1983; McManus and Lanier, 1987; Rohrbach et al., 1992; Jayarao and Henning, 2001; Jayarao et al., 2006). As part of the NAHMS Dairy 2014 study, thermophilic *Campylobacter* spp. (*C. jejuni*, *C. lari*, *C. coli*) were detected in BTM or filter samples from approximately one-quarter of a representative sample of dairy operations in the United States. This represents the first national survey to determine *Campylobacter* prevalence in BTM and milk filters in the United States.

In this study, *Campylobacter* spp. were detected in the filter samples but not in the BTM from 53 operations (15.1%, wp). Traditionally, BTM is sampled to assess milk quality and bacterial prevalence, and the purpose of the milk filter is to remove large debris, such as bedding, from the milk before storage in the bulk tank. The filter is not designed to remove bacterial pathogens, but bacterial cells may concentrate on the filter along with large debris. 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 bacterial pathogens in milk than BTM sampling alone (Van Kessel et al., 2008; Leone et al., 2010; Latorre et al., 2011; Van Kessel et al., 2011; Giacometti et al., 2012; Serraino et al.,

Table 5. Resistance of *Campylobacter lari* (n = 2) and *Campylobacter coli* (n = 1) isolates from bulk tank milk and milk filters from US dairies¹

Species	Resistance	No. of isolates	% of isolates
<i>C. lari</i>	Ciprofloxacin	2	100.0
	Nalidixic acid	2	100.0
<i>C. coli</i>	Susceptible ²	1	100.0

¹Resistance to azithromycin, ciprofloxacin, erythromycin, tetracycline, florfenicol, nalidixic acid, telithromycin, clindamycin, and gentamicin.

²Susceptible to all 9 antimicrobials.

Figure 2. Dendrogram of *Sma*I-digested pulsed-field gel electrophoresis patterns of *Campylobacter* spp. isolates (*C. jejuni*, *C. coli*, and *C. lari*) from bulk tank milk and milk filter pairs. TET = tetracycline, CIP = ciprofloxacin, and NAL = nalidixic acid; AR = antimicrobial resistance.

2013). Sampling filters in addition to BTM provides an additional opportunity to identify *Campylobacter* in milking systems.

Campylobacter spp. isolates were obtained from approximately half of the samples identified as positive via PCR, and, as expected, no isolates were obtained

from samples that were PCR-negative. Real-time PCR is widely considered to be a more sensitive means of detection than culture techniques, which may produce variable results. *Campylobacter* spp. are especially challenging to culture in the laboratory and require a microaerophilic environment, as well as supplementation with antimicrobials to inhibit competition from enteric flora. Although an operation-level prevalence of 12.5% (wp) via culture is similar to previous multi-state culture-based surveys of BTM (0.9–12.3%; Doyle and Roman, 1982; Lovett et al., 1983; McManus and Lanier, 1987; Rohrbach et al., 1992; Jayarao and Henning, 2001), reliance on culture techniques alone may result in a considerable underestimation of *Campylobacter* spp. prevalence due to the challenges in culture and isolation. Additionally, this survey was a single point-in-time sampling between March and July and may underestimate the actual prevalence of *Campylobacter*, because only 2 samples (BTM and milk filter) were taken per operation. Previous research indicates that *Campylobacter* spp. may be shed seasonally in dairy animals, with peaks in the spring in Wisconsin and in spring and fall in the United Kingdom (Stanley et al., 1998; Sato et al., 2004). Previous research into *Salmonella* also suggests that prevalence in BTM or filters may vary day to day, possibly due to variability in the events that lead to contamination of BTM (Van Kessel et al., 2012; Van Kessel et al., 2013). Previous national surveys based on fecal sampling of dairy cows have shown 90–100% of dairy operations are positive for *Campylobacter* spp., but these surveys collected 15–50 samples per operation, which increased the opportunity for detection of *Campylobacter* (USDA-APHIS, 2011). Therefore, although *Campylobacter* spp. have been detected in fecal samples from most dairy operations, the detection of *Campylobacter* in BTM is affected by the amount of fecal contamination in the system at the time of sampling.

The most frequent *Campylobacter* species associated with dairy animals, as well as human illness, is *C. jejuni* (CDC, 2014), and this was the predominant species isolated in the present study. Of the 49 isolates obtained from BTM and milk filter samples, 45 were *C. jejuni*; 1 *C. jejuni* isolate came from a milk filter from which *C. lari* was also isolated. Therefore, *C. jejuni* remains the *Campylobacter* species of greatest concern for the prevention and surveillance of foodborne disease.

An additional concern for foodborne pathogens is the potential for antimicrobial resistance. When we tested the susceptibility of *C. jejuni* isolates to a panel of common antimicrobials, 12 *C. jejuni* isolates (31.6%) were susceptible to all 9 antimicrobials, but 26 (68.4%) were resistant to tetracycline (Table 4). Although BTM and milk filters were not tested for the presence of *Campy-*

lobacter spp. in the NAHMS 2007 study, 62.4% of *C. jejuni* isolates from fecal samples in 2007 were resistant to tetracycline (USDA-APHIS, 2011). Tetracycline and its derivatives are used on dairy operations in milk replacers and footbaths, and bacterial resistance to this antimicrobial is common (Zwald et al., 2004; USDA-APHIS, 2011). Although campylobacteriosis in humans due to *C. jejuni* is usually self-limiting, erythromycin is the drug of choice when antimicrobial intervention is deemed necessary (Blaser and Engberg, 2008). All of the isolates collected in this survey were susceptible to erythromycin. Fluoroquinolones such as ciprofloxacin may also be used as an alternative to erythromycin, and a recent national survey of antimicrobial resistance in the United States identified resistance to ciprofloxacin in 22% of human *C. jejuni* isolates and 8.5% of dairy fecal isolates (CDC, 2014). We also observed resistance to ciprofloxacin in 5 isolates (13.2%) in this study.

The second most common cause of human *Campylobacter* infection is *C. coli*, although several other species are recognized as human pathogens, including *C. lari*. One *C. coli* isolate from a milk filter was susceptible to all tested antimicrobials, but we observed resistance to 2 quinolones (ciprofloxacin and nalidixic acid) in 2 *C. lari* isolates from milk filter samples. However, *C. lari* is an infrequent cause of human illness, and it is also considered intrinsically resistant to quinolones (Skirrow and Benjamin, 1980; CDC, 2016). Resistance to common antimicrobials did not appear to be a major attribute of the few *C. coli* and *C. lari* isolates in this survey; however, BTM may represent an additional source of pathogens, such as *C. jejuni*, that harbor antimicrobial resistance in the dairy farm environment.

When the *Sma*I restriction digest patterns were compared, *C. coli* and *C. lari* were distinct from the *C. jejuni* isolates, as expected (Figure 2). The *C. jejuni* strains were genotypically diverse, and clonal strains were geographically dispersed. When comparing the restriction digest patterns of isolates from BTM and filters, we found 9 indistinguishable *C. jejuni* clonal groups (100% similarity). Five of these clonal groups comprised isolates from operations in different states. In previous surveys of US dairy farms, Harvey et al. (2005) and Sanad et al. (2011) similarly described this genotypic similarity of isolates collected from different geographic regions. Dissemination across regions has been attributed to many sources, including migratory birds (Sanad et al., 2013; Keller and Shriver, 2014; Ryu et al., 2014). As observed in the present study and previous surveys (Sanad et al., 2011), *C. jejuni* isolates obtained from US dairy farms are genotypically diverse, and this diversity can extend to the farm level. On 2 occasions, different strains (<65% similarity) were isolated from a single operation. Given the high herd-level

prevalence on US dairy farms, this farm-level diversity could be due to the shedding of different strains by multiple cows in the milking herd. Alternatively, this could represent co-colonization by multiple strains in a single cow, as described by Nielsen (2002).

When comparisons included both restriction digest patterns and antimicrobial-resistant phenotypes, we observed no consistent association. Harvey et al. (2005) similarly observed that antimicrobial resistance patterns differed within some genotypes. In this study, resistance to tetracycline was dispersed throughout the 4 *C. jejuni* clades and across geographic regions (Figure 2). Although several indistinguishable *C. jejuni* isolate pairs exhibited similar resistance, in each of 2 pairs of indistinguishable isolates, 1 isolate was resistant to tetracycline and the second was susceptible, possibly attributable to several transmissible plasmid-encoded resistance genes, such as *tetO* (Taylor, 1986). Based on this and previous studies, antimicrobial resistance is not uncommon in *C. jejuni*, and phenotypic diversity in antimicrobial resistance may exist within clonal *C. jejuni* groups.

The CDC collects PFGE restriction digest patterns of pathogenic bacterial isolates from humans and other sources in the PulseNet database to identify foodborne outbreaks (Swaminathan et al., 2001). When we compared the PFGE patterns of the *Campylobacter* spp. isolates with the PulseNet database, 35 of 39 *C. jejuni* isolates matched pulsetypes isolated from humans in 2015, of which 7 were from raw milk. Study isolates also matched 22 isolates in the database that were obtained from poultry sources, such as chicken breasts, indicating that *Campylobacter* strains are not species-specific.

Campylobacter spp. are consistently found in feces of dairy animals in the United States and, in this study, were detected in BTM and milk filters from one-quarter of US dairy operations. As with other recent analyses of *Campylobacter* isolates and outbreak data, *C. jejuni* pulsetypes were commonly associated with those from PulseNet isolated from humans. Additionally, 68.4% of *Campylobacter* isolates were resistant to at least 1 antimicrobial. The estimated incidence of foodborne outbreaks due to raw milk consumption is over 150 times greater than the incidence due to pasteurized milk consumption (Langer et al., 2012), and *Campylobacter* spp. are the most common cause of these outbreaks. Therefore, contamination of BTM by *Campylobacter* spp. can lead to health risks for consumers of raw milk.

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