Prevalence and Antimicrobial Resistance of Thermophilic 
Campylobacter spp. from Cattle Farms in Washington State

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The prevalence of thermophilic Campylobacter spp. was investigated in cattle on Washington State farms. A total of 350 thermophilic Campylobacter isolates were isolated from 686 cattle sampled on 15 farms (eight dairies, two calf rearer farms, two feedlots, and three beef cow-calf ranches). Isolate species were identified with a combination of phenotypic tests, *hipO* colony blot hybridization, and multiplex lpaX PCR. Breakpoint resistance to four antimicrobials (*ciprofloxacin*, *nalidixic acid*, *erythromycin*, and doxycycline) was determined by agar dilution. *Campylobacter jejuni* was the most frequent species isolated (34.1%), followed by *Campylobacter coli* (7.7%) and other thermophilic campylobacters (1.5%). The most frequently detected resistance was to doxycycline (42.3% of 350 isolates). Isolates from calf rearer facilities were more frequently doxycycline resistant than isolates from other farm types. *C. jejuni* was most frequently susceptible to all four of the antimicrobial drugs studied (58.8% of 272 isolates). *C. coli* isolates were more frequently resistant than *C. jejuni*, including resistance to quinolone antimicrobials (89.3% of isolates obtained from calves on calf rearer farms) and to erythromycin (72.2% of isolates obtained from feedlot cattle). Multiple drug resistance was more frequent in *C. coli* (51.5%) than in *C. jejuni* (51.1%). The results of this study demonstrate that *C. jejuni* is widely distributed among Washington cattle farms, while *C. coli* is more narrowly distributed but significantly more resistant.

Members of the genus *Campylobacter* have long been recognized as a cause of septic abortion in both cattle and sheep, but the development and improvement of selective *Campylobacter* culture media led to the recognition that campylobacters can be etiological agent of human gastroenteritis (50). *Campylobacter* species are among the most frequently identified bacterial causes of human gastroenteritis in the United States (37) and other industrialized countries. In the United States, an estimated 2.4 million cases of human campylobacteriosis occur each year (18). Furthermore, there has been an increase in the frequency of resistance to drugs that are important in the treatment of human campylobacter gastroenteritis, especially fluoroquinolones and macrolides. Some groups have suggested that the increase in resistance among human *Campylobacter* isolates is attributable to transmission of antimicrobial-resistant animal isolates to humans (45, 46, 51).

The chicken is the species most frequently identified as a reservoir of bacteria responsible for human infection. Case-control studies have identified a significant association between *Campylobacter* infection in humans and handling and consumption of poultry (3, 10, 12, 27, 43). However, other studies have reported an association with cattle (2, 8, 19, 28–30, 41, 53, 54, 59, 61). Direct-contact exposure to bovine feces and ingestion of unpasteurized bovine milk are well-documented causes of outbreaks of campylobacteriosis (16, 44, 59). Recently, Fitzgerald et al. reported a high degree of genetic relatedness between the campylobacters from cattle and humans in the same geographical area (17). Because of the potential linkage between *Campylobacter* spp. harbored by cattle and human disease, in this study we investigated the prevalence, distribution, and antimicrobial resistance of thermophilic *Campylobacter jejuni*, *C. coli*, and other thermophilic *Campylobacter* (OTC) species in cattle.

**MATERIALS AND METHODS**

**Farm visits.** Fifteen cattle herds situated in the northwestern United States were selected for convenient access, including eight dairy farms, two calf rearer farms, two feedlots, and three beef cow-calf ranches based on the approximate proportion of each cattle farm type in the industry in Washington State. From February 2002 to April 2003, each operation was sampled twice at 6-month or longer intervals in order to increase the probability of *Campylobacter* sp. isolation.

**Samples.** On each farm visit, duplicate samples of rectal or free fecal dropings were obtained from 40 animals as follows: dairy farms and beef cow-calf ranches, calves 2 to 4 weeks old (*n = 20*) and adult recently fresh cows (*n = 20*); dairy calf rearers, calves 2 to 4 weeks old (*n = 40*); feedlots, cattle at 10 (*n = 20*) and 30 (*n = 20*) days on feed.

**Initial isolation and preservation of *Campylobacter* species.** Individual fecal specimens (10 g in 50-ml sterile plastic tubes) were collected from cattle and transported for processing within 6 h of the time of sampling. At the laboratory, a swab (approximately 0.1 g) of each fecal specimen was inoculated into Campy Thioglycolate medium (0.16% agar supplemented with trimethoprim, vancomy-
mum value section). C. jejuni to ensure inoculum delivery from all source wells of the 96-well plate and also for inoculation were described as resistant to that antimicrobial at the concentration included to validate assay performance. The plates were incubated microaerophilically at 37°C. Inocula that produced visible growth after 48 h of incubation were streaked for isolation on blood agar (Remel Inc.), incubated for 24 h at 42°C, and screened microscopically for typical Campylobacter morphology by Victoria Blue 4R staining. Presumptive thermophilic Campylobacter colonies were then suspended in Proteose Peptone (1% [wt/vol] glycerol (10% [vol/vol]) and stored at −70°C (55) for subsequent species identification and antimicrobial resistance determination.

Bacterial isolates and growth conditions. Campylobacter isolates were cultured on Columbia agar plates containing 5% sheep blood (Remel Inc.) in a microaerophilic atmosphere (Pack-MicroAero system; Mitsubishi Gas Chemical America, Inc., New York, N.Y.). All isolates were passed every 24 to 48 h. Escherichia coli and Staphylococcus aureus were cultured on Luria-Bertani agar plates (10 g of Bacto Tryptone per liter, 5 g of yeast extract per liter, 5 g of sodium chloride per liter, 15 g of Bacto Agar per liter; Becton Dickinson, Franklin Lakes, N.J.) in a 37°C incubator.

Antimicrobial susceptibility testing. Agar dilution antimicrobial susceptibility tests at breakpoint concentrations were performed with ciprofloxacin, nalidixic acid, erythromycin, and doxycycline (Sigma Chemical Co., St. Louis, Mo.) in accordance with NCCLS guidelines (38–40). Since no validated interpretive criteria for these four antimicrobials have been established for campylobacters, the breakpoints used by the Antimicrobial Resistance Research Unit, Agricultural Research Service, United States Department of Agriculture (http://www.arrs.usda.gov/narms.htm) were adopted for use in this study. Isolates were incubated microaerophilically at 37°C for 24 h in brain heart infusion (Becton Dickinson) broth (150 µl) in 96-well tissue culture plates (Becton Dickinson). A 96-pin replicator (Boekel Scientific, Feasterville, Pa.) was used to deliver ~10^5-CFU inocula of the enriched brain heart infusion broth cultures per pin to the following plates (all media were from Remel Inc.): (i) Mueller-Hinton agar with 5% defibrinated sheep blood (MHB; Remel Inc.), (ii) MHB containing ciprofloxacin (4 µg/ml), (iii) MHB containing nalidixic acid (32 µg/ml), (iv) MHB containing erythromycin (8 µg/ml), and (v) MHB containing doxycycline (16 µg/ml). With each assay batch, quality control strains (C. jejuni ATCC 33560, S. aureus ATCC 25923, S. aureus ATCC 29213, and E. coli ATCC 25922) were included to validate assay performance (36). The plates were incubated microaerophilically at 37°C. Inocula that produced visible growth after 48 h of incubation were described as resistant to that antimicrobial at the concentration in the agar plates. The inoculated MHB plates without antimicrobials were used to ensure inoculum delivery from all source wells of the 96-well plate and also for preparation of membrane blots used in the identification of C. jejuni (see next section).

Antimicrobial resistance to the four antimicrobials was codified with a binary code indicating resistant and susceptible as 1 and 0, respectively. Within isolates from specific farm types, resistance indices (RI) were calculated by dividing the sum of codified resistance of all isolates by the total number of isolates (maximum value = 4).

Identification of thermophilic Campylobacter spp. For specific identification of C. jejuni, a digoxigenin-labeled oligonucleotide probe specific for the hipcRNA-encoding gene (hipO) of C. jejuni was prepared with primers Hip100-F and Hip1128-R (4) and the PCR DIG synthesis kit (Roche, Mannheim, Germany). For hipO colony hybridization, the protocol described in the DIG application manual for filter hybridization (Roche website: http://www.roche-applied-science.com) was adopted, with minor modifications of the hybridization temperature and high-stringency wash temperature (overnight hybridization at 41°C and washes at 65°C). A positive chemiluminescence signal was regarded as an indication of C. jejuni, and a negative signal was regarded as an OTC species. The chromosomal DNA was extracted from 24-h blood agar plate cultures of all hipO-negative isolates with the DNaseasy tissue kit (QIAGEN, Valencia, Calif.). DNA concentrations were measured on a spectrophotometer, and the preparations were stored at −20°C. Next, hipO-negative isolates were tested with a multiplex PCR to identify C. jejuni, C. coli, C. lari, and C. upsaliensis (60). For isolates that were not identified by these two methods, a cephPCR assay specific for C. coli and 16S rRNA sequencing were also performed (23, 34). Isolates that grew well at 42°C but tested negative by hipO hybridization, negative by multiplex PCR, and negative by cephPCR and finally identified only by 16S rRNA sequencing were identified in this paper as OTC species.

RESULTS

Characterization of isolates. The major phenotypic characteristics of the isolates obtained in this study were typical of thermophilic Campylobacter spp. All isolates demonstrated spiral or curved rod morphology in Victoria blue staining, and all of the isolates tested exhibited oxidase and catalase activity and reduced nitrate. Aerobic growth was not observed, and bacterium was hydrolyzed by putative C. jejuni isolates. All C. jejuni and C. coli isolates were positive by either hipO DNA probe hybridization or multiplex species identification PCR with the appropriate primer sets.

Prevalence of thermophilic campylobacters. A total of 686 fecal specimens (172 from beef cow-calf ranches, 105 from calf rearer operations, 311 from dairy farms, and 98 from feedlots) were sampled and analyzed for campylobacters. At least one animal was positive for thermophilic Campylobacter sp. on each farm. One or more isolates of C. jejuni, C. coli, and OTC species was isolated from 234 (34.1%), 53 (7.7%), and 10 (1.5%) of the fecal samples, respectively (Table 1). The prevalence by herd type was significantly different for both C. jejuni and C. coli (P < 0.001), with C. jejuni isolates at a higher prevalence in beef cow-calf ranches than in other herd types.

<table>
<thead>
<tr>
<th>Herd type</th>
<th>No. of samples tested</th>
<th>No. (%) of Campylobacter-positive samples*</th>
<th>Concurrent excretionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef cow-calf</td>
<td>172</td>
<td>C. jejuni 81* (47.1) C. coli 11 (0.6) Other species 3 (1.7)</td>
<td>Type 1 4 (2.3) Type 2 2 (1.2)</td>
</tr>
<tr>
<td>Calf rearer</td>
<td>105</td>
<td>C. jejuni 25† (23.8) C. coli 21* (20.0) Other species 0 (0)</td>
<td>Type 1 4 (3.8) Type 2 6 (5.7)</td>
</tr>
<tr>
<td>Dairy</td>
<td>311</td>
<td>C. jejuni 97† (31.2) C. coli 189 (5.8) Other species 5 (1.6)</td>
<td>Type 1 10 (3.2) Type 2 18 (5.8)</td>
</tr>
<tr>
<td>Feedlot</td>
<td>98</td>
<td>C. jejuni 31† (31.6) C. coli 13* (13.3) Other species 2 (2.0)</td>
<td>Type 1 7 (7.1) Type 2 7 (7.1)</td>
</tr>
<tr>
<td>Total</td>
<td>686</td>
<td>C. jejuni 234 (34.1) C. coli 53 (7.7) Other species 10 (1.5)</td>
<td>Type 1 25 (3.6) Type 2 33 (4.8)</td>
</tr>
</tbody>
</table>

* Herd types with different (P < 0.05) prevalences of Campylobacter spp. are indicated by different superscripts within a column.

b Concurrent excretion in this study is defined as a single fecal sample harboring multiple Campylobacter species (type 1) or distinct isolates of a single Campylobacter species with different antimicrobial resistance phenotypes (type 2).
and *C. coli* isolates at a higher prevalence in calf rearer farms and feedlots than in other farm types. More than one *Campylobacter* species or more than one isolate of a single *Campylobacter* species with differing antimicrobial resistance phenotypes (concurrent excretion) was detected in 58 (8.5%) of the fecal samples. A somewhat higher frequency of concurrent excretion was detected in fecal samples from feedlot cattle, although this difference was not statistically significant (*P*/H11022 < 0.05). Including the multiple isolates resulting from concurrent excretion, a total of 350 thermophilic *Campylobacter* sp. isolates were investigated: 272 of *C. jejuni*, 66 of *C. coli*, and 12 of OTC species (Table 2). On the basis of 16S rRNA sequences, the OTC species were most similar to *C. jejuni* (*n*/H11005 = 4), *C. coli* (*n*/H11005 = 4), *C. hyointestinalis* (*n*/H11005 = 1), and *C. fetus* (*n*/H11005 = 3). On the basis of their divergent *hipO* hybridization or PCR, *lpxA* PCR, and *ceuE* PCR results, these additional *C. jejuni* and *C. coli* isolates were identified as OTC species.

**Antimicrobial resistance.** The most frequently detected resistance was to doxycycline (42.3% of 350 isolates). The majority of *C. jejuni* isolates (160 [58.8%] of 272) were susceptible to the four antimicrobials drugs screened. Of the 112 *C. jejuni* isolates with detected antimicrobial resistance, 107 (39.3%), 8 (2.9%), and 14 (5.1%) were resistant to doxycycline, erythromycin, and quinolones, respectively. The frequency of doxycycline-resistant *C. jejuni* isolates was highest (21 [80.8%] of 26; *P* < 0.001) on calf rearer farms, although larger numbers of doxycycline-resistant *C. jejuni* strains were isolated from dairy farm cattle (46 [43.0%] of 107). Multiple antimicrobial drug resistance was relatively rare in the *C. jejuni* isolates in this study (14 [12.5%] of 112 resistant isolates) (Fig. 1).

*C. coli* isolates were more frequently (*P* < 0.001) antimicrobial resistant than *C. jejuni* isolates were (Table 2). Specifically, 48 (72.7%) *C. coli* isolates were resistant to one or more of the antimicrobial drugs tested, including 37 (56.1%), 21 (31.8%), and 30 (45.5%) with resistance to doxycycline, erythromycin, and quinolones, respectively. Calf rearer farm isolates accounted for 68% (25 of 37) of the doxycycline-resistant and 83% (25 of 30) of the quinolone-resistant isolates. Feedlot cattle isolates accounted for 62% (13 of 21) of the erythromycin-resistant isolates (Fig. 2). Multiple drug resistance was more frequent (*P* < 0.001) in *C. coli* (34 [51.5%] of 66 isolates) than in *C. jejuni* (14 [5.1%] of 272 isolates).

RI were calculated, and the values were compared among the different thermophilic *Campylobacter* spp. and among the

| Herd type         | *C. jejuni* | *C. coli* | Other species | Overall
<table>
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<tr>
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<tbody>
<tr>
<td>Beef cow-calf</td>
<td>0.27* (22/83)</td>
<td>0 (0/1)</td>
<td>0.67 (2/3)</td>
<td>0.28† (24/87)</td>
</tr>
<tr>
<td>Calf rearer</td>
<td>0.81† (21/26)</td>
<td>2.89† (81/28)</td>
<td>0 (0/0)</td>
<td>1.89† (102/54)</td>
</tr>
<tr>
<td>Dairy</td>
<td>0.49‡ (60/122)</td>
<td>0.42* (8/19)</td>
<td>0.57 (4/7)</td>
<td>0.49* (72/148)</td>
</tr>
<tr>
<td>Feedlot</td>
<td>0.68‡§ (28/41)</td>
<td>1.33‡ (24/18)</td>
<td>1.00 (2/2)</td>
<td>0.89§ (54/61)</td>
</tr>
<tr>
<td>Avg</td>
<td>0.48 (131/272)</td>
<td>1.71 (113/66)</td>
<td>0.67 (8/12)</td>
<td>0.72 (252/350)</td>
</tr>
</tbody>
</table>

*RI is the sum of codified resistance of total isolates divided by the total number of isolates (maximum value = 4). Herd types with different (*P* < 0.05) Campylobacter sp. RI are indicated by different symbols within a column.*

FIG. 1. Number of *C. jejuni* isolates by resistance and herd type. PS, pansensitive; D, doxycycline resistant; E, erythromycin resistant; N, nalidixic acid resistant; C, ciprofloxacin resistant.
different herd types in this study (Table 2). The RI was highest for *C. coli* isolated from calf rearer farms than for isolates from other farm types.

**DISCUSSION**

In general, the occurrence of human campylobacter gastroenteritis has been largely attributed to the consumption of contaminated food animal products, especially poultry, because of the high prevalence of campylobacters in these animals (3, 10, 12, 27, 43). A growing body of evidence, however, suggests that other vehicles such as red meat, environmental water, and unpasteurized milk may be important sources of these organisms (19, 25, 44).

The media and isolation conditions used in this study were primarily developed for isolating *C. jejuni* and *C. coli*, and this may have biased detection in favor of these two species (2, 5, 11, 15). Nevertheless, the prevalence of thermophilic campylobacters (*C. jejuni*, 34%; *C. coli*, 8%; other, 2%) in this study is generally concordant with the previous studies in which the prevalence of campylobacters in cattle ranged from 0.8 to 46.7%, depending on the isolation methods, herd size and type, geography, season, animal age, and number of animals investigated (9, 21, 28, 41, 47, 61).

Recently, Sato et al. (49) reported that 27.9% of fecal specimens from dairy herds in Wisconsin were positive for *Campylobacter* spp. on the basis of a culture method that did not include the Campy Thioglycolate selection used in this study. Although recovery of *Campylobacter* spp. is reduced by temporary storage of fecal specimens at 4°C for 24 h (33), as was necessary in our study, we nevertheless obtained a higher isolation rate than did Sato et al. Differences in the sampling, storage, and bacteriological methods between these two studies preclude conclusions about the relative prevalences of cattle excreting thermophilic *Campylobacter* spp. in their feces.

We isolated *C. jejuni* at the highest prevalence in our beef cow-calf study herds, whereas *C. coli* was relatively more prevalent in calf rearer and feedlot farm types. The concurrent excretion frequency we determined in this study (9%) is lower than that (24%) reported by Inglis et al. (31). However, we did not attempt to discriminate genetically different strains within multiple isolates of the same *Campylobacter* sp. with identical resistance patterns and so probably underestimated the actual frequency of concurrent fecal excretion by the cattle in this study.

Twelve putative thermophilic campylobacters were finally identified as *C. jejuni*, *C. coli*, *C. hyointestinalis*, and *C. fetus*. The misidentification of these *C. jejuni* isolates could be attributable to alteration or loss of the *hipO* gene, alteration of the *lpxA* gene, or other, unknown, contributing factors. Since *lpxA* encodes UDP-GlcNAc acyltransferase, an enzyme required for lipid A biosynthesis (56), partial mutation of the *lpxA* gene in the primer annealing region is considered more likely than deletion of the gene. Given the composition of isolation media and the isolation conditions used, isolation of *C. hyointestinalis* is plausible and has been observed by other investigators (2, 8, 21, 31). Although the isolation of thermotolerant *C. fetus* was fortuitous, other investigators have reported the isolation of similar atypical *C. fetus* strains from human and raw milk (13, 32, 62). Although the hybridization method used in this study resulted in several false-negative signals in the screening for *C. jejuni*, the overall sensitivity of this method (261 [94%] of 278 *C. jejuni* isolates) was robust.

In human campylobacteriosis, *C. coli* is reported less frequently than *C. jejuni*. Recent case-control studies suggest that the etiological risk factors for human infection with *C. coli* differ from those for infection with *C. jejuni* and that the health burden attributable to *C. coli* is considerable and more significant in terms of public health than previously thought (22, 35, 57).
Ciprofloxacin, nalidixic acid, erythromycin, and doxycycline were chosen for antimicrobial susceptibility testing in this study because of their importance as front-line therapeutic drugs in humans (ciprofloxacin, erythromycin), the relatively frequent occurrence of resistance to these drugs (24), and the published validated methods for these drugs (36, 38, 40). Validated methods have been established for other drugs in addition to the four drugs tested, but previous studies have suggested that resistance to these additional drugs was very rare (Centers for Disease Control and Prevention National Antimicrobial Resistance Monitoring System website; http://www.cdc.gov/narms).

Resistance to the antimicrobials included in this survey was more prevalent in \textit{C. coli} than in \textit{C. jejuni}, similar to observations by others on human isolates, as well as on food animal isolates (20, 24, 58). \textit{C. jejuni} isolates demonstrated a markedly lower rate of multiple drug resistance (Fig. 1) than did \textit{C. coli} (Fig. 2) or OTC species (data not shown).

The most frequent resistance was to doxycycline (42%), which is consistent with that reported by Sato et al. (49). Doxycycline resistance in thermophilic \textit{Campylobacter} spp. has been attributed to a \textit{tet} resistance gene carried on a conjugative plasmid (52).

The frequency of resistance to erythromycin was higher among \textit{C. coli} isolates (31.8%) than among \textit{C. jejuni} (2.9%) or OTC species (0%) isolates in this study, as others have previously reported (1, 20, 58). Several antimicrobial drugs frequently used in cattle populations may have selected for erythromycin resistance in these herds, including spiramycin and erythromycin (used to treat bovine mastitis [14]) or tyllosin (used to prevent the formation of hepatic abscesses in feedlot cattle and occasionally used in other food animal species for therapeutic purposes [9, 26]). Erythromycin resistance was found only at a low frequency in \textit{C. jejuni} or \textit{C. coli} isolated from broilers in which tyllosin had not been used as a growth promoter (48).

The frequency of resistance to ciprofloxacin was higher in \textit{C. coli} isolates (44%) than in OTC species (25%) or \textit{C. jejuni} (5%) isolates. Relatively higher levels of resistance to fluoroquinolone antimicrobials in \textit{C. coli} isolated from food animal have also been reported in other studies (20, 58). Unlike the solely ciprofloxacin-resistant \textit{Campylobacter} spp. reported by Sato et al. (49), 1 \textit{C. jejuni} isolate and 25 \textit{C. coli} isolates with resistance to ciprofloxacin were also resistant to doxycycline or erythromycin, which suggests that the development of antimicrobial resistance might have been multifactorial in this study rather than only a point mutation in antimicrobial resistance determinants.

It was not possible to obtain accurate data on the frequency of use and the types of antimicrobial drugs used on the farms in this study because of the limited number and duration of the farm visits. Collection of accurate drug use data in large operations like these study herds would require intensive longitudinal efforts, including acquisition of animal treatment records, interviews with multiple personnel with animal treatment responsibilities, acquisition of records of feed and drug purchases, and consultation with one to several veterinarians per farm to collect and analyze current and historical antimicrobial drug prescription records. Nevertheless, the results of this study support the need to perform such studies, since the marked differences in the frequency of antimicrobial resistance of thermophilic \textit{Campylobacter} sp. isolates by farm type are suggestive of differences in the degree of antimicrobial selection pressures by farm type.

The results of this study demonstrate that \textit{C. jejuni} is widely distributed among northwestern U.S. cattle farms, while \textit{C. coli} is more narrowly distributed and is especially frequent at calf rearer farms. The distribution of antimicrobial resistance in thermophilic \textit{Campylobacter} spp. may result from increased antimicrobial drug selection pressures on calf rearer farms compared to other farm types. The frequent isolation of multidrug-resistant \textit{C. coli} from the farms in this study suggests that \textit{C. coli} is more likely to acquire resistance or that resistant strains of this species are more likely to be widely disseminated than \textit{C. jejuni} strains.

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