High prevalence of fluoroquinolone-resistant Campylobacter in sheep and increased Campylobacter counts in the bile and gallbladder of sheep medicated with tetracycline in feed

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High prevalence of fluoroquinolone-resistant Campylobacter in sheep and increased Campylobacter counts in the bile and gallbladder of sheep medicated with tetracycline in feed

Abstract

Campylobacter is a major foodborne pathogen in humans and a significant cause of abortion in sheep. Although ruminants are increasingly recognized as important reservoirs for Campylobacter, limited information is available about the molecular epidemiology and antimicrobial resistance (AMR) profiles of sheep Campylobacter. Here we describe a two-trial study that examined Campylobacter profiles in sheep and determined whether in-feed tetracycline influenced the distribution and AMR profiles of Campylobacter. Each trial involved 80 commercial sheep naturally infected with Campylobacter, 40 of which were medicated with tetracycline in feed, while the other 40 received feed without antibiotics. Fecal and bile samples were collected for the isolation of Campylobacter. The bacterial isolates were analyzed for antimicrobial susceptibility and genotypes. The results revealed that 87.0% and 61.3% of the fecal and bile samples were positive for Campylobacter (C. jejuni and C. coli), with no significant differences between the medicated and non-medicated groups. All but one of the tested Campylobacter isolates were resistant to tetracycline. Although fluoroquinolone (FQ) resistance remained low in C. jejuni (1.7%), 95.0% of the C. coli isolates were resistant to FQ. Genotyping revealed that C. jejuni ST2862 and C. coli ST902 were the predominant genotypes in the sheep. Feed medication with tetracycline did not affect the overall prevalence, species distribution and AMR profiles of Campylobacter, but increased the total Campylobacter counts in bile and gallbladder. These findings identify predominant Campylobacter clones, reveal the high prevalence of FQ-resistant C. coli, and provide new insights into the epidemiology of Campylobacter in sheep.

Keywords

Campylobacter, sheep, antimicrobial resistance, genotype, fluoroquinolone

Disciplines

Large or Food Animal and Equine Medicine | Veterinary Infectious Diseases | Veterinary Microbiology and Immunobiology

Comments


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High prevalence of fluoroquinolone-resistant *Campylobacter* in sheep and increased *Campylobacter* counts in the bile and gallbladder of sheep medicated with tetracycline in feed

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Running title: *Campylobacter* in sheep

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Campylobacter is a major foodborne pathogen in humans and a significant cause of abortion in sheep. Although ruminants are increasingly recognized as important reservoirs for Campylobacter, limited information is available about the molecular epidemiology and antimicrobial resistance (AMR) profiles of sheep Campylobacter. Here we describe a two-trial study that examined Campylobacter profiles in sheep and determined whether in-feed tetracycline influenced the distribution and AMR profiles of Campylobacter. Each trial involved 80 commercial sheep naturally infected with Campylobacter, 40 of which were medicated with tetracycline in feed, while the other 40 received feed without antibiotics. Fecal and bile samples were collected for the isolation of Campylobacter. The bacterial isolates were analyzed for antimicrobial susceptibility and genotypes. The results revealed that 87.0% and 61.3% of the fecal and bile samples were positive for Campylobacter (C. jejuni and C. coli), with no significant differences between the medicated and non-medicated groups. All but one of the tested Campylobacter isolates were resistant to tetracycline. Although fluoroquinolone (FQ) resistance remained low in C. jejuni (1.7%), 95.0% of the C. coli isolates were resistant to FQ. Genotyping revealed that C. jejuni ST2862 and C. coli ST902 were the predominant genotypes in the sheep. Feed medication with tetracycline did not affect the overall prevalence, species distribution and AMR profiles of Campylobacter, but increased the total Campylobacter counts in bile and gallbladder. These findings identify predominant Campylobacter clones, reveal the high prevalence of FQ-resistant C. coli, and provide new insights into the epidemiology of Campylobacter in sheep.

**Keywords:** Campylobacter, sheep, antimicrobial resistance, genotype, fluoroquinolone
Importance

*Campylobacter* is a major cause of foodborne illness in humans and antibiotic-resistant

*Campylobacter* is considered a serious threat to public health in the United States and worldwide. As a foodborne pathogen, *Campylobacter* commonly exists in the intestinal tract of ruminant animals such as sheep and cattle. Results from this study reveal the predominant genotypes and high prevalence of tetracycline and fluoroquinolone resistance in sheep *Campylobacter*. The finding on fluoroquinolone resistance in sheep *Campylobacter* is unexpected as this class of antibiotics is not used for sheep in the United States, and it may suggest the transmission of fluoroquinolone-resistant *Campylobacter* from cattle to sheep. Additionally, the results demonstrate that in-feed medication with tetracycline increases *Campylobacter* counts in gallbladders, suggesting that the antibiotic promotes *Campylobacter* colonization of gallbladder. These findings provide new information on *Campylobacter* epidemiology in sheep, which may be useful for curbing the spread of antibiotic-resistant *Campylobacter* in animals reservoirs.

Introduction

*Campylobacter* species, particularly *Campylobacter jejuni* and *Campylobacter coli*, are a leading cause of bacterial foodborne gastroenteritis in humans around the world (1-3). Among all the causes of laboratory-confirmed bacterial food-borne illnesses, *Campylobacter* was the leading cause (19.2 per 100,000 population) in the year of 2017 based on the report by the Centers for Disease Control and Prevention (CDC)’s FoodNet surveillance program in the United States (4). Although most *Campylobacter*-related illnesses in humans are characterized as self-limiting diarrhea (watery and/or bloody), antibiotic treatment is utilized in more severe
clinical conditions, especially in young, elderly, or immunocompromised patients (5). However, antimicrobial resistance in *Campylobacter* is increasingly prevalent and has become a major public health concern in both developed and developing counties (6, 7). Of particular concern is the rising resistance to fluoroquinolone (FQ) and macrolide antibiotics, which are clinically important for the treatment of *Campylobacter* induced diarrhea in humans, but in some cases, tetracyclines and gentamicin are also used to treat systemic infection caused by *Campylobacter* (6, 8, 9).

Food-producing animals are important reservoirs for *Campylobacter* (10). Epidemiologically, poultry meat is considered the major source of infection for human campylobacteriosis (11). *C. jejuni* and *C. coli* frequently colonize the intestines of many species of poultry, especially commercial chickens and turkeys, usually as part of the normal microbial flora without causing clinical disease (12). Recently, the role of ruminants (cattle and sheep) in *Campylobacter* ecology has been increasingly recognized in different countries (13-17). Ruminant *Campylobacter* can be transmitted to humans via contaminated milk and water, environmental contamination or direct contact with animals (18). Source attribution studies indicate that ruminant *Campylobacter* is a significant source of infection for human campylobacteriosis (19-21).

Typically, *Campylobacter* colonizes in the intestinal tract in animals, but it may also translocate across the intestinal epithelial barrier and causes systemic infection, such as bacteremia and abortion in ruminants and even in humans occasionally (1, 22). In fact, *Campylobacter* is the major cause of ovine abortions worldwide, including the U.S. Notably, a single hypervirulent tetracycline-resistant *C. jejuni* clone (named clone SA for sheep abortion) is responsible for the majority of *Campylobacter*-associated ovine abortions in the U.S. (17, 23, 24).
Previous survey of healthy sheep in a slaughterhouse revealed genetically diverse *C. jejuni* strains (including clone SA) present in the intestinal tract and gallbladder (25). Despite the fact that ruminants are increasingly recognized as important reservoirs for *Campylobacter*, limited information is available about the molecular epidemiology and antimicrobial resistance profiles of sheep *Campylobacter* in the United States.

For the control of abortion and other diseases, the tetracycline class of antibiotics is commonly used in the U.S., and it is the only class of antibiotics approved for prevention and control of sheep abortion associated with *Campylobacter* (23). However, whether the administration of tetracycline in feed impacts the prevalence of *Campylobacter* in sheep is not known. Previously, it was shown that sheep harbored diverse strains of *C. jejuni* that were commonly resistant to tetracycline; however, resistance to FQ was seldomly detected (25).

Recently, FQ resistance in cattle *Campylobacter* isolates has increased substantially in the U.S. (15, 26-28). However, detection of FQ resistance in sheep *Campylobacter* isolates is still limited.

To address these knowledge gaps, we collected samples and isolated *Campylobacter* from controlled treatment (feed medication with tetracycline) studies using sheep that were derived from commercial operations and were naturally infected by *Campylobacter*. The goals were to examine the extent of FQ resistance in sheep *Campylobacter* and determine the effect of in-feed tetracycline on the prevalence, species distribution, and antimicrobial resistance profiles of *Campylobacter* in sheep.

**Materials and Methods**

*Sample collection and Campylobacter isolation*

Two separate trials were conducted and each involved 80 feeder lambs, with 40 each in the feed medicated group and feed non-medicated group. These sheep were purchased from two
commercial farms and then housed in the laboratory animal facility at Iowa State University during the treatment study. Trial 1 was conducted using animals from farm 1, while trial 2 was performed using animals from Farm 2. The two farms are in the Midwest region and are geographically separated by approximately 86 miles. The ages of the sheep at enrolment varied, but all were spring-born feeder lambs and approximately two months old. Both source farms raise sheep in a semi-intensive management program with outdoor access. Treatment of sheep with FQ antibiotics is expressly prohibited in the U.S. and there was no history of use of these antibiotics on these farms.

In each trial, the animals were randomly assigned into the feed medicated and non-medicated groups. Chlortetracycline (Aureomycin, Zoetis) was used for the feed medication, and the medication was conducted to determine the effect of the antibiotic on prevention and control of bacterial pneumonia in feedlot lambs. The antibiotic was fed extra-label (350 mg/hd/d; 160 mg/kg body weight or 160 ppm in feed) in accordance with the FDA/CVM’s Guidance 615.115. Regardless of the feed medication, clinically sick animals (19 in each group for trial 1; 17 and 18, respectively, for trail 2) received a chlortetracycline injection (9 mg/lb) and the injection repeated once in 72 hr if needed. The treated sheep remained in their respective groups.

Trial 1 was performed from June 21, 2017 to July 6, 2017, while Trial 2 was carried out from July 13, 2017 to July 26, 2017 (Table 1). Fecal samples were collected at the beginning of the study and once a week thereafter. Bile and gallbladder samples were collected at necropsy. The samples were then cultured for _Campylobacter_ using Mueller–Hinton (MH) agar plates (Difco) with selective antimicrobials and growth supplements (SR084E and SR117E; Oxoid). The plates were incubated at 42 °C for 48 h under microaerobic conditions (85% N2, 5% O2 and 10% ...
CO2). Up to three *Campylobacter*-like colonies from each sample were subcultured onto MH agar plates and the pure cultures were stored in glycerol stocks at −80 °C.

**Enumeration of Campylobacter in bile and gallbladder mucosa**

In addition to isolation of *Campylobacter* from individual bile samples as described above, we also determined the total *Campylobacter* colony forming units (CFUs) in bile and gallbladder mucosa. For this purpose, bile and gallbladder samples were randomly collected from sheep at necropsy in the two trials. In total, 37 and 35 bile/gallbladder mucosal samples were selected in the two trials from the feed non-medicated and feed medicated groups, respectively. For each group, the sheep were housed in 4 separate pens and the collected bile from each gallbladder (5-15 ml) was pooled based on treatment group and pen number. In total, 4 pools were obtained per group in each trial. For collection of gallbladder mucosal samples, 5 ml of PBS was used to wash any residual bile off each gallbladder mucosa. Then, a sterile razor blade was used to scrape mucosa from each gallbladder onto a bile-free weigh boat. Next, the blade was washed with PBS to rinse any mucosa on the blade onto the weigh boat, which then was rinsed with roughly 2 ml of PBS, and the collected gallbladder mucosa was decanted into a 15 ml conical tube. The final volume was adjusted to 3 ml, and the 15 ml conical tube was vortexed vigorously prior to culture for *Campylobacter*.

For both the bile and gallbladder mucosal samples, a ten-fold dilution series was performed using 1.5 ml snap cap tubes filled with 900 μl of MH broth. Each dilution was spread onto MH agar plates containing selective supplement (SR084E; Oxoid) and standard culture conditions for *Campylobacter* were utilized as for the fecal samples (described above). After culturing, the *Campylobacter* CFUs were counted for each sample and the Log10 average was computed for both bile and gallbladder mucosa respectively. The detection limit of the plating method was 100
CFU/mL of bile or gallbladder mucosal suspension. All of the 16 pooled bile samples demonstrated visually pure growth of *Campylobacter* sp. and were utilized in the final calculations. For the gallbladder mucosal samples, 4 in the non-medicated group and 9 in the medicated group were not usable because of high background bacterial contamination after culturing. The remaining plates were visually determined to have pure growth of *Campylobacter* sp. and thus were used in the final calculations. In total, *Campylobacter* CFU counts were obtained from 33 and 26 gallbladder mucosal samples in the non-medicated and medicated groups, respectively. Representative colonies from each group were selected and subjected to species confirmation and identification (see below).

**Bacterial species identification**

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker) was used for bacterial species identification. All isolates were identified to the species level. Sample preparation and analysis were done as described previously (29). Mass spectra were acquired and analyzed using a microflex LT mass spectrometer (Bruker Daltonics) in combination with research-use-only version of the MALDI Biotyper Compass software 4.1 and the reference database MBT 7311 MSP Library (#1829023) at Iowa State University. Data were interpreted in accordance with the manufacturer’s (Bruker Daltonics) standard criteria: a) high-confidence identification when the score was between 2.00 and 3.00, b) low-confidence identification when the score was between 1.70 and 1.99, and c) no organism identification possible when the score was 1.69 and lower.

**C. jejuni Clone SA detection**

PCR was used for identifying *C. jejuni* clone SA. DNA template was extracted from *Campylobacter* colonies using single-cell lysis buffer (30). Primers specific for *C. jejuni* clone
SA were designed as described previously (31). C. jejuni IA3902, a clinical isolate of clone SA, was used as the positive control, while a reaction without template DNA served as a negative control.

Antimicrobial susceptibility testing

A total of 236 C. jejuni and 101 C. coli non-duplicate isolates from fecal samples were tested for antimicrobial susceptibility. Those isolates were representatives of individual animals at different sampling times. Also, 98 C. jejuni and 2 C. coli non-duplicate isolates (a single isolate from each animal) from bile samples were included in the susceptibility testing. The minimum inhibitory concentrations (MICs) of nine antibiotics were determined using a standard broth microdilution method as recommended by CLSI and the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) (32-34). The tested ranges and breakpoints of the nine antibiotics were listed in Table S1. Commercially available Sensititre Campylobacter plates (ThermoFisher Scientific, Waltham, MA) were used for the tests. The nine antibiotics were azithromycin (AZI), ciprofloxacin (CIP), erythromycin (ERY), gentamicin (GEN), tetracycline (TET), florfenicol (FFN), nalidixic acid (NAL), telithromycin (TEL) and clindamycin (CLI). After incubation in a microaerobic environment for 24 h at 42 °C, the results were interpreted. For each isolate, MIC values were set as the lowest antimicrobial concentration at which no bacterial growth was observed. The antimicrobial resistance breakpoints were chosen according to the standards established by the CLSI for bacteria isolated from animals (32-34). C. jejuni ATCC 33560 and C. coli ATCC 33559 were used as quality control strains, respectively.

PFGE and MLST typing of Campylobacter isolates
Representative *Campylobacter* isolates were analyzed by using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE analysis of the macrorestriction fragment patterns of genomic DNA using *Kpn*I enzyme was performed following the CDC’s standardized PulseNet protocol for *Campylobacter* with minor modifications (23). Briefly, fresh cultures of *Campylobacter* were embedded in 1% Seakem Gold agarose (Fisher Scientific, Fair Lawn, NJ) and lysed with proteinase K for 30 minutes at 50 °C in a water bath shaker. The gel plugs were digested with *Kpn*I for 4 h at 37 °C. Digested plugs were embedded into 1% agarose and separated by electrophoresis in 0.5 × TBE buffer (Promega) at 14 °C for 18 h using a Chef Mapper electrophoresis system (Bio-Rad, Hercules, CA). The gels were stained with ethidium bromide for 30 min and then photographed using a digital imager (Alpha Innotech, Santa, Clara, CA). The PFGE patterns were analyzed by the GelCompare II v.6.5 software (Applied Maths, Kortrijk, Belgium) using Dice similarity coefficient and unweighted-pair group method with arithmetic averages (UPGMA) with 0.5% optimization and 1.5% position tolerance. Lambda DNA ladder (Bio-Rad) was used as the molecular size marker. PFGE patterns with ≥90% similarity was defined as a cluster.

To further confirm the genetic diversity of the isolates, MLST was performed for selected representative isolates of different PFGE patterns as described previously (35). The seven housekeeping genes were amplified and sequenced using the primers recommended by the *Campylobacter* MLST website (https://pubmlst.org/campylobacter/), which was developed by Keith Jolley and Man-Suen Chan at the University of Oxford (36). All PCR products were purified using the QIAquick® PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced at the DNA Core Facility of Iowa State University using an Applied Biosystems 3730xl DNA Analyzer. The sequences were submitted to the MLST database to determine allele
numbers and STs. The new allele and STs were submitted to the database and were assigned new numbers.

gyrA mutation determination

In *Campylobacter*, FQ resistance is conferred by point mutations in the *gyrA* gene in conjunction with the CmeABC efflux pump (37). To confirm the mechanism of FQ resistance in this study, four FQ<sup>R</sup> *C. jejuni* isolates and eight FQ<sup>R</sup> *C. coli* isolates of different PFGE and MLST types were selected for determination of the point mutations in the quinolone resistance determining region (QRDR) of *gyrA*. The primers GyrAF1 (5′-CAACTGGTTCTAGCCTTTTG-3′) and GyrAR1 (5′-AATTTCACTCATAGCCTACG-3′) were used for *C. jejuni* isolates, while GyrAF2 (5′-TTATTTAGATTATTCTATGAGCGT-3′) and GyrAR2 (5′-CTTGAGTTCGATTACAACAC-3′) were used for *C. coli* isolates as described previously (15).

All PCR products were purified using the QIAquick® PCR purification kit (QIAGEN, Hilden, Germany) and then sequenced at the DNA Core Facility of Iowa State University using an Applied Biosystems 3730xl DNA Analyzer. Statistical analysis

Chi-square test and Fisher’s exact test were used to compare the prevalence and the antimicrobial resistance rates of the *Campylobacter* isolates from different samples (fecal and bile samples) and different groups (feed non-medicated and medicated groups). Unpaired t-test was used to compare the average *Campylobacter* CFU/ml results from the bile and gallbladder mucosal samples between the non-medicated and medicated groups in two trials. The data was analyzed using GraphPad (Prism). *P* values less than 0.05 were deemed to be statistically significant.
**Results**

*Prevalence of Campylobacter in sheep*

In total, 461 sheep fecal samples and 160 bile samples were collected for isolation of *Campylobacter* in the two separate trials. The overall prevalence rate of *Campylobacter* in sheep feces was 87.0% (401/461). Among the positive samples, 68.6% (275/401) and 37.4% (150/401) were positive for *C. jejuni* and *C. coli*, respectively, and 24 fecal samples were positive for both *C. jejuni* and *C. coli* (Table 1). The overall prevalence of *Campylobacter* in fecal samples was comparable (71.8% vs. 65.3% for *C. jejuni*; 33.2% vs. 41.7% for *C. coli*) between the feed medicated and non-medicated groups (*p* > 0.05). (Table 1). Among the bile samples, 61.3% (98/160) were positive for *Campylobacter* (Table 2). Of the *Campylobacter* isolates, 98.0% (96/98) were *C. jejuni*, and 2.0% (2/98) were *C. coli*. The two *C. coli* isolates from bile were both isolated from the feed medicated group in Trial 2 (Table 2). Again, there was no significant difference (*p* > 0.05) between the groups in the prevalence of either *C. jejuni* (100% vs. 95.8%) or *C. coli* (0% vs. 4.2%). These results indicate that the in-feed tetracycline medication did not affect the overall prevalence of *Campylobacter* in fecal and bile samples.

Statistically, the overall *Campylobacter* prevalence was higher in fecal samples than in bile samples (87.0% vs. 61.3%, *p* < 0.05). However, the proportion of *C. jejuni* was significantly (*p* < 0.05) lower in feces than in bile (68.6% vs. 98.0%), while the proportion of *C. coli* was higher in feces than in bile (37.4% vs. 2.0%). Detailed information about the prevalence of *Campylobacter* and the distribution of *C. jejuni* and *C. coli* is summarized in Tables 1 and 2. We analyzed all of the *C. jejuni* isolates from feces and bile by PCR for identification of *C. jejuni* clone SA, and none of them were positive, indicating clone SA was not present in the sheep used in this study.
Quantitative measurement of Campylobacter CFUs in bile and gallbladder mucosa

Significant differences in Campylobacter numbers (CFU/ml) between the feed medicated and non-medicated groups were detected for both the bile and gallbladder mucosal samples, with numbers higher in the medicated group (Figure 1). In the bile samples, the mean logCFU/ml ± SEM (standard error of the mean) were 5.041±0.281 and 6.021±0.108 in non-medicated and medicated group, respectively, indicating an almost 10-fold difference. The difference between the two groups was statistically significant \[t(3.252,6) = 0.0174, P < 0.05\]. For the gallbladder mucosal samples, the mean logCFU/ml ± SEM of gallbladder mucosal samples were 4.7239±0.2932 and 5.823±0.2611 in the non-medicated and medicated groups, respectively, again indicating an approximately 10-fold difference. The difference was also statistically significantly \[t(2.722,57) = 0.0086, P < 0.05\]. These results indicated that Campylobacter titers are higher in the gallbladder and bile samples taken from the feed medicated group.

Antimicrobial susceptibility of the Campylobacter isolates

For the tested fecal C. jejuni isolates (n=236), all (100%) were resistant to tetracycline, but the resistance rates to ciprofloxacin and nalidixic acid were low, at 1.7% (4/236) and 3.0% (7/236), respectively. All four ciprofloxacin-resistant (CIP\textsuperscript{R}) C. jejuni isolates were derived from Trial 2. One C. jejuni isolate from the non-medicated group in Trial 1 showed resistance to gentamicin. The fecal C. coli isolates also showed a high resistance rate to tetracycline (99.0%; 100/101), with only one sensitive to this antibiotic. In contrast to C. jejuni, the C. coli isolates were highly resistant to ciprofloxacin (95.0%; 96/101) and nalidixic acid (95.0%; 96/101). Resistance to the other five antibiotics were not observed. The key MIC results are shown in Table 3, while other relevant MIC data (MIC ranges, MIC\textsubscript{50}, and MIC\textsubscript{90}) were presented in Table S1.
For the isolates from bile samples, all tested *C. jejuni* were resistant to tetracycline (100.0%, 96/96), but susceptible to other antibiotics, while the two *C. coli* isolates were resistant to tetracycline, ciprofloxacin and nalidixic acid. None of the bile *C. jejuni* or *C. coli* isolates showed resistance to gentamicin, azithromycin, erythromycin, florfenicol, telithromycin or clindamycin.

Notably, the CIP resistance rate (95.0%) in *C. coli* is significantly (*P* < 0.05) higher than in *C. jejuni* (1.7%) for the fecal samples. There is no significant difference (*P* > 0.05) in the overall antimicrobial susceptibility profiles as tested in this study between fecal and bile *C. jejuni*. The two *C. coli* isolates from bile samples also showed similar resistance patterns to the *C. coli* isolates from feces. In addition, there was also no significant difference (*P* > 0.05) in the antimicrobial resistance rates of *C. jejuni* and *C. coli* between the feed non-medicated and feed medicated groups.

**Genetic diversity of the Campylobacter isolates from sheep**

In total, 24 *C. coli* and 39 *C. jejuni* isolates were selected for PFGE analysis. The twenty-four *C. coli* consisted of twelve CIP\(^R\) C. coli from feces (one representative isolate for each group and each sampling time in the two trials), the two CIP\(^R\) *C. coli* isolates from bile, the five CIP\(^S\) fecal *C. coli*, the TET susceptible *C. coli* and the other four fecal *C. coli* (isolated from the same sheep with the two CIP\(^R\) *C. coli* isolates from bile). The 24 *C. coli* isolates were grouped into three separate clusters, with the vast majority of isolates (75.0%, 18/24) grouped into type III (Figure 2). The five CIP\(^S\) *C. coli* isolates were all in type I, and type II included one CIP\(^R\) *C. coli* from feces. The predominated cluster (type III) consisted of 18 CIP\(^R\) *C. coli*, among which 16 were from fecal samples (including one TET susceptible isolate and 4 from the same sheep where the two CIP\(^R\) bile isolates were obtained) and two from bile samples (Figure 2). MLST analysis
showed that the *C. coli* isolates grouped in type Ⅰ, Ⅱ, Ⅲ belong to ST827, ST1068 and ST902, respectively (Figure 2), consistent with the classifications using PFGE. Thus, ST902 was the most prevalent ST type in the sheep *C. coli* analyzed in this study. Interestingly, the CIP\(^R\) bile and fecal isolates from the same sheep shared the same PFGE type (III) and MLST type (ST902), indicating the bile and fecal isolates are genetically related.

The 39 *C. jejuni* included twelve CIP\(^S\) *C. jejuni* from bile (three representative isolates for each group in two trials), the four CIP\(^R\) *C. jejuni* from feces, the three CIP\(^S\) fecal *C. jejuni* (isolated from the same sheep with the CIP\(^R\) isolates), twenty CIP\(^S\) fecal *C. jejuni* isolates (from different sheep that CIP\(^R\) isolates were isolated, representative isolates from each group and each sampling time in two trials, and including ten selected fecal *C. jejuni* isolated from the same sheep with the six bile *C. jejuni* isolates). The 39 *C. jejuni* isolates were clustered into five different clusters (Figure 3), apart from four isolates that were non-typeable by PFGE because they were not digestible by KpnI. The four non-typeable isolates included one CIP\(^S\) bile isolate and three selected CIP\(^S\) fecal isolates from the same three sheep with bile isolates. Type A is the predominant genotype and accounted for 69.2% (27/39) of the analyzed isolates. This predominant cluster include both CIP\(^R\) and CIP\(^S\) *C. jejuni* isolated from fecal and bile samples. MLST results indicated that all but two *C. jejuni* isolates in type A belonged to ST2862 (Figure 2). One of the two non-ST2862 isolates was a CIP\(^R\) *C. jejuni* (ID: 0993), which showed only one base difference in *gltA* with ST2862. After submitting the sequence to the MLST database, the new *gltA* allele was assigned to *gltA*586 (in comparison it is *gltA*2 in ST2862) and the isolate was assigned a new ST type ST9380. The other non-ST2862 isolate was also a new ST type (ST9379) and was a bile isolate (ID: 292). The detailed information of MLST profiles in the two isolates were presented in Table S2. The other four PFGE patterns, types B, C, D and E,
consisted of 8 *C. jejuni* isolates. Type B belonged to ST982, while types C, D, and E all belonged to ST52 (Figure 3). For the selected bile and fecal *C. jejuni* isolates from the same sheep, the situation was more diverse comparing with the *C. coli* isolates (Figure 3). Bile and fecal isolates from each of the animals with an ID number 898, 346 and 604 shared the same PFGE and MLST profiles, while the bile and fecal isolates from other three animals were of different PFGE or MLST types (ID292, the same PFGE with different ST types; ID961, different PFGE types but with the same ST type; ID610, different PFGE types with different ST types).

Altogether, the PFGE and MLST findings indicated predominant genotypes in both *C. coli* and *C. jejuni*, suggesting clonal expansion may have been involved in the dissemination of both *C. coli* and *C. jejuni* in sheep.

**Point mutations in gyrA**

Based on different PFGE and MLST types, eight CIP\(^R\) *C. coli* and four CIP\(^R\) *C. jejuni* isolates were selected for determination of the point mutations in gyrA. All the CIP\(^R\) *C. coli* isolates harbored a single Thr-86-Ile mutation in GyrA without any other amino acids changes in this region (Figure 2). For the CIP\(^R\) *C. jejuni* isolates, three had the Thr-86-Ile point mutation alone, and one carried the Thr-86-Ile mutation plus the Ser-22-Gly, Asn-203-Ser and Arg-285-Lys changes (Figure 3). These results are consistent with the known role of the Thr-86-Ile mutation in mediating CIP resistance in *Campylobacter*.

**Discussion**

Results from this study revealed high prevalence of TET-resistant *Campylobacter* spp. and CIP\(^R\) *C. coli* in sheep derived from two commercial farms. These animals were naturally infected and already carried antibiotic-resistant *Campylobacter* on the day of arrival (Tables 1, 2, and 3). Subsequent treatment with in-feed tetracycline in a research setting did not affect the overall
prevalence, antimicrobial susceptibility profiles, and species distribution of Campylobacter as there were no statistically significant differences ($p > 0.05$) between the feed medicated and non-medicated groups. However, the quantitative CFU counts in bile and gallbladder mucosa collected at the end of the trials revealed higher total CFUs in the medicated group than the non-medicated group ($P < 0.05$), suggesting that tetracycline treatment may promote Campylobacter colonization in gallbladder. The exact reason for this effect is unknown, but it is known that tetracyclines undergo significant enterohepatic circulation, resulting in significant concentration of tetracyclines in bile, which may inhibit competitive bacterial organisms and confer a colonization advantage for tetracycline-resistant Campylobacter. Since similar numbers of animals received therapeutic treatment in both groups, it is likely that the effect is attributable to in-feed use of tetracycline. Regardless of the reasons, the enhanced colonization of gallbladder may give Campylobacter an advantage in the spread to other organs or dissemination back to the intestinal tract.

In fecal samples, C. jejuni accounted for 68.6% of the total isolates (Table 1), while in bile samples the isolates were almost exclusively C. jejuni (98.0%), suggesting that C. jejuni has a higher tropism to bile or a higher ability to reach to and adapt within the gallbladder. Previous studies observed similar prevalence between C. jejuni and C. coli in sheep gallbladders (38, 39), however, these previous studies were conducted in a different country and it is possible that differences in sheep production practices or genetic variability of Campylobacter strains may influence gallbladder colonization by the organism. Bile is harmful to bacteria, however, Campylobacter utilizes the multidrug efflux pump CmeABC for bile resistance (40-43). The exact reason for the predominance of C. jejuni in gallbladder as observed in this study is unknown and remains to be investigated in future studies. Interestingly, C. jejuni clone SA was
not detected in the sheep examined in this study. This clone is a predominant cause of
Campylobacter-associated ovine abortions and is also distributed in feedlot and dairy cattle in
the U.S. (23, 44). A recent study also reported identification of Clone SA in Grenada (45). The
lack of clone SA detection in this study is not entirely surprising as we have previously found
that the distribution of clone SA is highly variable from farm to farm (25). Thus, it was likely
that the two farms where the sheep were derived from did not harbor this particular C. jejuni
strain.

Except for FQs and tetracycline, the Campylobacter isolates examined in this study were
generally susceptible to other tested antimicrobials (Table 3). All but one of the tested
Campylobacter isolates from fecal and bile samples were resistant to tetracycline. This finding
further strengthens the result from our previous study on Campylobacter carriage in sheep
slaughterhouse, which revealed the resistance rate to tetracycline was already 83.3% (25). In this
study, the in-feed tetracycline medication did not further influence the prevalence of tetracycline-
resistant Campylobacter due to the fact that tetracycline resistance in the sheep was already very
high (virtually 100%) at the beginning of the treatment. Thus, the result should be interpreted
precautiously. Given the high prevalence of tetracycline-resistant Campylobacter on sheep farms
in the U.S., the use of tetracycline as a mean to control Campylobacter-induced sheep abortion
would have a limited impact. In fact, tetracycline medication may have facilitated the persistence
of tetracycline-resistant Campylobacter on sheep farms. Results from this study further underline
the need for improved tetracycline stewardship in sheep to reduce the prevalence of tetracycline-
resistant Campylobacter.

A significant finding of this work is the detected high prevalence of CIPR C. coli (95%),
while CIP resistance in the sheep C. jejuni isolates remained low (1.7%) (Table 3). The
difference between C. coli and C. jejuni is unlikely due to variation in their susceptibility to fluoroquinolones as CIP\textsuperscript{R} C. jejuni is also common in other animal species (6, 7). The high-level prevalence of CIP\textsuperscript{R} C. coli in sheep was unexpected as this class of antibiotics is not used in sheep production in the United States. A recent study on cattle Campylobacter reported CIP resistance rates of 35.4\% in C. jejuni and 77.3\% in C. coli (15). This could be explained by the fact that FQ antibiotics are labeled for treatment of respiratory disease in cattle in the U.S. The reason for the detected high incidence of CIP\textsuperscript{R} C. coli in sheep in this study is unknown, but there is a possibility of transmission of CIP\textsuperscript{R} Campylobacter from cattle to sheep. Although the sampled sheep farms were not adjacent to other animal species, they could get Campylobacter through birds, flies, or other transmission vehicles. However, if this were the sole case, we would have also observed an increased prevalence of CIP\textsuperscript{R} C. jejuni in sheep as it is also common in cattle. It is possible that the CIP\textsuperscript{R} C. coli strains were introduced to sheep by chance and something on these farms favored the selection of CIP\textsuperscript{R} C. coli, contributing to its overall high prevalence in sheep. It should be pointed out that fluoroquinolones are also approved for use in swine and were used in poultry prior to 2005 in the U.S. Thus, the possibility that sheep acquired CIP\textsuperscript{R} C. coli from other animal species rather than cattle can't be totally excluded.

To determine the genetic relatedness of the Campylobacter isolates, we conducted PFGE and MLST analyses, which are commonly used for genotyping of Campylobacter (20, 46-48). PFGE typing of 24 representative C. coli revealed a limited number of types and that the majority (18/24) of the CIP\textsuperscript{R} C. coli isolates were grouped into a single type (type III; Figure 2), which was confirmed by MLST to be a single ST (ST902). It is worthwhile to note that type III included isolates from different and geographically distant farms and from different sampling times, suggesting that this genotype is stable and is prevalent on different sheep farms. Also, bile
and fecal isolates from the same sheep shared the same PFGE type and ST type. The other two
PFGE types belonged to ST827 (type I) and ST1068 (type II). C. coli ST827 has been previously
reported, sometimes to be the most common ST in both sheep and cattle (49, 50), while ST1068
is more often found in cattle (15, 50, 51). Moreover, ST1068 was also identified among swine C.
coli isolates, sometimes to be the most predominant ST type (51–53). In addition, the three
identified STs (ST902, ST827 and ST1068) all belong to the ST-828 complex (Figure 2).
Although the specific prevalent ST types vary in different species, C. coli isolates of ST-828
complex, the most prevalent CC (clonal complex) for C. coli, have been frequently reported in
published studies from different countries and different sources, including poultry, cattle, sheep,
swine and even humans (15, 54–57). Our results confirmed the prevalence of this CC and the
existence of specific endemic ST type among animals in different regions.
The 39 representative C. jejuni isolates examined by PFGE typing in this study, they were
grouped into five clusters (Figure 3), with the exception of four that were not typeable by PFGE
because KpnI failed to digest appropriately. Similar to the C. coli result, the majority (27/39) of
the tested C. jejuni isolates was grouped into one cluster (type A), which was confirmed by
MLST to be a single ST (ST2862) except for two isolates, suggesting it was the predominant C.
jejuni clone in sheep. This clone included both CIP$^R$ and CIP$^S$ C. jejuni isolated from both fecal
and bile samples that were collected from different farms (trials 1 and 2) and different sampling
times (Figure 3), indicating this genotype was commonly disseminated in sheep. The other four
PFGE types belonged to ST982 (type B) and ST52 (types C, D and E). ST982 accounted for
20.8% (10/48) of the C. jejuni isolates in a previous study involving a lamb slaughterhouse and
was also reported in cattle (15, 25, 58). ST-52 was reported sporadically in C. jejuni from
different countries and sources, including ruminants (45-47, 57, 59, 60). Both ST2862 and ST982, belong to CC21, which is the most prevalent CC in *C. jejuni*.

To examine the possible transmission of *Campylobacter* between sheep and cattle, we selected some cattle *Campylobacter* isolates identified in our previous study (15) to compare with the sheep *Campylobacter* isolates identified in this study. The PFGE and MLST results revealed that ST902 (the predominant *C. coli* genotype in sheep identified in this study) with the same PFGE pattern was also found in the cattle *C. coli* (Figure S1a), although the predominant ST was ST1068 in cattle (15). Here, we also identified ST1068 in sheep *C. coli* (Figure 2), which had a similar PFGE pattern with cattle *C. coli* (Figure S1b). These findings further suggest the possible transmission of FQ-resistant *C. coli* (e.g. ST902 and ST1068) between cattle and sheep. In contrast to the *C. coli* isolates, the predominant *C. jejuni* genotype ST2862 (Figure 3) in sheep was not previously identified in cattle in the U.S. (15, 26, 58). Although *C. jejuni* ST982 has been found in cattle (15), their PFGE patterns were quite different from the sheep isolates (Figure S1c). Thus, the genetic relatedness between sheep and cattle *C. jejuni* isolates were not as clear as for *C. coli*.

In *Campylobacter*, FQ-resistance is mediated by point mutations in the QRDR of DNA gyrase (GyrA) and the function of the CmeABC multidrug efflux pump (8, 61). The most frequent mutation associated with FQ resistance in *Campylobacter* is Thr-86-Ile, followed by Asp-90-Asn, Thr-86-Lys, Thr-86-Ala, Thr-86-Tyr, and Ala-70-Val (8, 62). Additionally, double mutations including Thr-86-Ile/Pro-104-Ser and Thr-86-Ile/Asp-90-Asn have also been associated with FQ resistance in *Campylobacter* (63). The mutation of Asn-203-Ser is also known to confer FQ resistance along with the Thr-86-Ile mutation (64). Ser-22-Gly has not been linked to FQ resistance in *Campylobacter* and there was no evidence that Asn-203-
Ser and Arg-285-Lys alone were associated with the FQ resistance phenotype (15). In this study, all examined FQ\textsuperscript{R} Campylobacter isolates harbored the Thr-86-Ile mutation in GyrA, while one isolate also carried the Ser-22-Gly, Asn-203-Ser and Arg-285-Lys mutations, consistent with the known role of the Thr-86-Ile change (alone or along with other mutations) in CIP resistance.

In summary, we observed high prevalence of tetracycline-resistant Campylobacter and FQ\textsuperscript{R} C. coli in commercial sheep operations, regardless of in-feed medication with tetracycline. Although the medication increased the total Campylobacter CFU counts in the sheep gallbladder, it did not affect overall prevalence, genotypes, or antimicrobial susceptibility profiles of the Campylobacter isolates under the conditions employed in this study. The sheep harbored predominant genotypes of C. jejuni (ST2862) and C. coli (ST902). Of particular note, the FQ\textsuperscript{R} C. coli clones in the sheep (both ST902 and ST1068) were genetically linked to the isolates previously identified in cattle, suggesting the possibility of dynamic transmission of FQ\textsuperscript{R} C. coli between sheep and cattle. However, there are several limitations in this study. First, the work was a controlled laboratory treatment study, and the pre-existing high prevalence of tetracycline-resistant Campylobacter limited our ability to analyze the effect of the in-feed medication on development of tetracycline resistance. Second, the sheep were derived only from two commercial farms and the sample sizes were not particularly large. Thus, the findings may not represent the overall situation with sheep Campylobacter in the U.S. Despite these limitations, the findings provide new and useful information on sheep Campylobacter. Since ruminants are important reservoirs for Campylobacter and a significant source of foodborne campylobacteriosis in humans, enhanced efforts should be directed toward the development of interventions to reduce prevalence and transmission of FQ\textsuperscript{R} Campylobacter in sheep and cattle.
Acknowledgements

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Figure 1. *Campylobacter* counts (Log10 CFUs/ml) in bile (a) and gallbladder mucosal (b) samples in the non-medicated and medicated groups. Each bar represents the average log10 CFUs per mL (+/- SEM). "*" indicates significant difference from the non-medicated group (P < 0.05). The data include samples from both trials.

Figure 2 PFGE patterns of representative *C. coli* isolates in sheep. T1 and T2: Trials 1 and 2; W1, W2, and W3: 1st, 2nd and 3rd week; CIP: ciprofloxacin. NC: at necropsy; ND: not done. #: The TET susceptible *C. coli*. The bold fonts in animal IDs indicate the animals from which both bile and fecal isolates were typed.

Figure 3 PFGE patterns of representative *C. jejuni* isolates in sheep. T1 and T2: Trials 1 and 2; W1, W2, and W3: 1st, 2nd and 3rd week; CIP: ciprofloxacin. NC: at necropsy; ND: not done. *indicated newly assigned ST types. The bold fonts in animal IDs indicate the animals from which both bile and fecal isolates were typed. Four of the *C. jejuni* isolates were non-typeable by PFGE and are not shown.
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<td>8</td>
<td>9</td>
<td>17</td>
<td>13</td>
<td>10</td>
<td>23</td>
</tr>
</tbody>
</table>

NM: non-medicated group; M: medicated group; T: total. ²4 fecal samples were positive both for *C. jejuni* and *C. coli.*
Table 2. Overall prevalence of *Campylobacter* in sheep bile samples*

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Trial 1 (6/21/2017-7/6/2017)</th>
<th>Trial 2 (7/13/2017-7/26/2017)</th>
<th>All bile samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Groups</td>
<td>NM</td>
<td>M</td>
</tr>
<tr>
<td>No. isolates (%)</td>
<td></td>
<td>28 (50.9)</td>
<td>27 (49.1)</td>
</tr>
<tr>
<td>C. jejuni (%)</td>
<td></td>
<td>28 (50.9)</td>
<td>27 (49.1)</td>
</tr>
<tr>
<td>C. coli (%)</td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

NM: non-medicated group; M: medicated group; T: total. *Bile samples were collected at necropsy at the end of the two trials.
Table 3 Antimicrobial resistance profiles and rates of the tested *Campylobacter* isolates from fecal samples

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Tetracycline</th>
<th>Ciprofloxacin</th>
<th>Nalidixic acid</th>
<th>Gentamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-medicated</td>
<td>100.0% (60/60)</td>
<td>0.0% (0/60)</td>
<td>3.3% (2/60)</td>
<td>1.7% (1/60)</td>
</tr>
<tr>
<td>Medicated</td>
<td>100.0% (60/60)</td>
<td>0.0% (0/60)</td>
<td>5.0% (3/60)</td>
<td>0.0% (0/60)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>100.0% (120/120)</strong></td>
<td><strong>0.0% (0/120)</strong></td>
<td><strong>4.2% (5/120)</strong></td>
<td><strong>0.8% (1/120)</strong></td>
</tr>
<tr>
<td><strong>C. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-medicated</td>
<td>100.0% (25/25)</td>
<td>100.0% (25/25)</td>
<td>100.0% (25/25)</td>
<td>0.0% (0/25)</td>
</tr>
<tr>
<td>Medicated</td>
<td>100.0% (25/25)</td>
<td>100.0% (25/25)</td>
<td>100.0% (25/25)</td>
<td>0.0% (0/25)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>100.0% (50/50)</strong></td>
<td><strong>100.0% (50/50)</strong></td>
<td><strong>100.0% (50/50)</strong></td>
<td><strong>0.0% (0/50)</strong></td>
</tr>
<tr>
<td><strong>Trial 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0% (118/118)</strong></td>
<td><strong>94.0% (107/115)</strong></td>
<td><strong>94.0% (107/115)</strong></td>
<td><strong>0.0% (0/118)</strong></td>
</tr>
<tr>
<td><strong>C. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-medicated</td>
<td>96.0% (24/25)</td>
<td>88.0% (22/25)</td>
<td>88.0% (22/25)</td>
<td>0.0% (0/25)</td>
</tr>
<tr>
<td>Medicated</td>
<td>100.0% (26/26)</td>
<td>92.3% (24/26)</td>
<td>92.3% (24/26)</td>
<td>0.0% (0/26)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>98.0% (50/51)</strong></td>
<td><strong>90.2% (46/51)</strong></td>
<td><strong>90.2% (46/51)</strong></td>
<td><strong>0.0% (0/51)</strong></td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>100.0% (138/138)</strong></td>
<td><strong>94.0% (131/140)</strong></td>
<td><strong>94.0% (131/140)</strong></td>
<td><strong>0.0% (0/138)</strong></td>
</tr>
<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-medicated</td>
<td>100.0% (118/118)</td>
<td>0.8% (1/118)</td>
<td>3.4% (4/118)</td>
<td>0.8% (1/118)</td>
</tr>
<tr>
<td>Medicated</td>
<td>100.0% (118/118)</td>
<td>2.5% (3/118)</td>
<td>2.5% (3/118)</td>
<td>0.0% (0/118)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>100.0% (236/236)</strong></td>
<td><strong>1.7% (4/236)</strong></td>
<td><strong>3.0% (7/236)</strong></td>
<td><strong>0.4% (1/236)</strong></td>
</tr>
<tr>
<td><strong>C. coli</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-medicated</td>
<td>98.0% (49/50)</td>
<td>94.0% (47/50)</td>
<td>94.0% (47/50)</td>
<td>0.0% (0/50)</td>
</tr>
<tr>
<td>Medicated</td>
<td>100.0% (51/51)</td>
<td>96.1% (49/51)</td>
<td>96.1% (49/51)</td>
<td>0.0% (0/51)</td>
</tr>
<tr>
<td>Total</td>
<td><strong>99.0% (100/101)</strong></td>
<td><strong>95.0% (96/101)</strong></td>
<td><strong>95.0% (96/101)</strong></td>
<td><strong>0.0% (0/101)</strong></td>
</tr>
</tbody>
</table>