Prevalence, Enumeration, and Antimicrobial Agent Resistance of Clostridium difficile in Cattle at Harvest in the United States

ALEXANDER RODRIGUEZ-PALACIOS,1,2 MOHAMMAD KOOHMARAIE,3 AND JEFFREY T. LEJEUNE1,2,*

1Food Animal Health Research Program, College of Food, Agricultural, and Environmental Sciences, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691; 2Department of Preventive Veterinary Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, Ohio 43210; and 3Institute for Environmental Health Laboratories and Consulting, Lake Forest Park, Washington 98155, USA

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ABSTRACT

To assess the potential for food contamination with Clostridium difficile from food animals, we conducted a cross-sectional fecal prevalence study in 944 randomly selected cattle harvested at seven commercial meat processing plants, representing four distinct regions (median distance of 1,500 km) of the United States. In all, 944 animals were sampled in the summer of 2008. C. difficile was isolated from 1.8% (17 of 944) of cattle, with median fecal shedding concentration of 2.2 log CFU/g (range = 1.6 to 4.8, 95% confidence interval = 1.6, 4.3). Toxigenic C. difficile isolates were recovered from only four (0.4%) cattle. One of these isolates was emerging PCR ribotype 078/toxinotype V. The remaining toxigenic isolates were toxinotype 0, one of which was an isolate with resistance to linezolid, clindamycin, and moxifloxacin (by the E-test). All isolates were susceptible to vancomycin, metronidazole, and tigecycline, but the MICs against linezolid were as high as the highest reported values for human-derived isolates. The source of the linezolid-clindamycin-moxifloxacin resistance in a toxigenic C. difficile isolate from cattle is uncertain. However, since the use of these three antimicrobial agents in cattle is not allowed in North America, it is possible that resistance originated from an environmental source, from other species where those antimicrobial agents are used, or transferred from other intestinal bacteria. This study confirms that commercial cattle can carry epidemiologically relevant C. difficile strains at the time of harvest, but the prevalence at the time they enter the food chain is low.

Clostridium difficile has been isolated from food animals and retail meats (including ground beef) since 2006, raising concerns about potential zoonotic and foodborne transmission of this pathogen (34, 35). Despite the increasing number of published studies on the subject, most research about C. difficile in food producing animals has focused on determining the prevalence of fecal shedding in very young stock (19, 24, 28, 34), an animal population that contributes minimally (~0.5%) to the millions of pounds of meat processed for human consumption in the United States every year (46).

During processing of ground beef, carcass trimmings from mature animals are mixed to achieve target fat contents between 3 and 30% in final products. While fat trimmings often originate from cattle intentionally raised for beef production (hereinafter referred to as “fed” cattle), the sources of leaner trimmings are frequently mature cattle primarily used for breeding purposes or milk production (hereinafter referred to as “mature” cattle), which can be domestic or imported (23). Both types of cattle represent distinct production systems, might have different risks and prevalence of pathogens, and therefore could differentially contribute to microbial contamination of ground beef products.

At the time of harvest, fecal contamination of the hides and processing facilities contaminate carcasses (9). Establishing the fecal prevalence of C. difficile in cattle at harvest is thus a valuable step in assessing the potential risk for food contamination (carcass or meat products) in the early stages of food processing. This study determined the prevalence and magnitude of fecal shedding of C. difficile in commercial fed and mature cattle at harvest in the United States. Furthermore, we assessed the public health relevance of recovered isolates by characterizing their toxin genes and antimicrobial agent resistance patterns to six drugs of relevance in human medicine.

MATERIALS AND METHODS

Meat processing plants. We conducted a cross-sectional study of C. difficile in fed and mature cattle at the time of harvest at seven commercial beef processing plants in four regions of the United States. The establishments were inspected by the U.S. Department of Agriculture, harvested between 400 and 1,500 cattle per day, and were sampled once during the summer (July to September) of 2008. Five of these participants specialized in harvesting either fed or mature cattle, whereas the other two processed both animal types.

Sampling of animals. Animals sampled were from commercial lots scheduled for harvest the day of sampling and they were sampled during the normal harvest process. The geographical

* Author for correspondence. Tel: 330-263-3739; Fax: 330-263-3677; E-mail: lejeune.3@osu.edu.
distribution of the plants was deemed adequate to represent the diversity of cattle production systems that ship animals for slaughter in the United States, including the eastern, central, western, and southern regions. Three of the seven meat processing plants indicated that animals arriving from Canada were also being harvested on the day of sampling. In the processing line, sampling was conducted by dedicated personal working at the conveyor belt immediately after each evisceration station. In plants where fed and mature cattle were both harvested, the plant supervisor guided the sampling by indicating the respective viscera to the sampling personnel. Based on systematic sampling principles, animals were sampled at regular animal intervals to minimize potential clustering associated with cattle groupings or lots. The intervals were calculated by dividing the number of animals available for harvest the day of sampling over the estimated sample size.

Starting with the first harvested animals, and continuing consecutively for 5 to 6 h (50 to 80% of required time to process all animals), 10 g of fecal contents was aseptically obtained through a 10- to 15-cm incision made on the dorsal wall of the rectum, about 20 cm from the anus. Fecal samples were stored in Whirl-Pak bags (Nasco, Fort Atkinson, WI) and shipped refrigerated to The Ohio State University for C. difficile testing. Within 24 h of collection, samples were coded and blindly processed. Aliquots from the first two processing plants (western region) were frozen at −80°C to determine the magnitude of fecal shedding (i.e., CFU enumeration) later.

**Enrichment culture and molecular typing.** C. difficile prevalence was based on an enrichment protocol used in previous studies (2, 34, 36). In brief, 1 g of manually homogenized feces was enriched in 15 mL of selective C. difficile broth (2) supplemented with 0.1% sodium taurocholate (Sigma Aldrich, St. Louis, MO) and C. difficile supplement SR0096 (broth concentrations of cycloserine and cefoxitin were 250 and 8 mg/L, respectively; Oxoid, Ltd., London, UK). After anaerobic incubation at 37°C for 7 days (anaerobic chamber, CO₂:H₂:N₂ gas ratio of 10:10:80) and centrifugation of the broths (7,000 × g for 10 min), the sediments were treated with 96% ethanol (1:1 [vol/vol], 30 min), centrifuged again to remove the supernatants, and plated onto 3-h prerduced C. difficile fructose agar (CM0601, Oxoid, Ltd.) supplemented with 7% defibrinated horse blood (Quad Five, Ryegate, MT) and the cycloserine-cefoxitin supplement. After 5 days of anaerobic incubation at 37°C, up to three suspect yellow-green fluorescent (UV₅₆₅) colonies were subcultured onto tryptic soy agar (Acumedia, Lansing, MI) supplemented with 5% defibrinated sheep blood (Quad Five). Nonhemolytic isolates, positive for l-proline aminopeptidase activity (Pro Disc, Remel, Lenexa, KS) (10) were stored at −80°C for molecular confirmation.

Frozen isolates were revived on blood agar, and after 48 h of anaerobic incubation, single colonies were processed for DNA extraction with a commercial Chelex resin (InstaGene Matrix, Bio-Rad, Hercules, CA). C. difficile was molecularly characterized by using three multiplex PCR reactions. One multiplex confirmed C. difficile by detecting the triosephosphate isomerase gene (tpi) and toxin genes tcdA and tcdB (21) and the second by detecting a typical 16S rRNA gene amplicon and toxin genes tcdA, tcdB, cdtA, and cdtB (26); the third multiplex assessed the presence of the putative toxin regulator gene tcdE by using tcdE as a referent amplicon (13, 42). PCR ribotyping and visual comparison of resulting fingerprint patterns were used to assess strain similarity among cattle types and regions (3). In addition, toxotyping (i.e., restriction fragment length polymorphism of the pathogenicity locus) was conducted on toxin gene fragments A3 and B1, as described by M. Rupnik (http://www.mf.uni-mb.si/mikro/tox/). Isolates were deemed toxigenic if they had at least one of the tcdA, tcdB, cdtA, and cdtB toxin genes and associated toxins (37).

**Toxin production.** In addition to identifying the toxin genes, representative isolates were tested with two complementary commercial enzyme-linked immunosorbent assays (ELISAs) to assess their toxigenic potential, as described previously (14, 45), by using 72-h culture colonies. Toxin gene testing was conducted at least in duplicate with reference toxigenic strain ATCC 9569 (tcdA⁺, tcdB⁺, cdtA⁺, cdtB⁺, toxinnotype 0) as positive control. The production of C. difficile toxins A and B was confirmed by interpreting in parallel ELISA results from commercial kits C. difficile TOX A/B II (TechLab, Blacksburg, VA) and Clearview C. diff A (Wampole Laboratories, Inverness Medical Professional Diagnostics, Princeton, NJ). Binary toxin production was not evaluated.

**Dialysis tubing and toxin cytotoxicity assay on Vero cells.** To confirm the lack of toxin production by strains identified as toxin negative by PCR and ELISA, selected isolates were tested in dialysis assays (43) with toxigenic strain ATCC 9569 and nontoxigenic C. difficile PCR ribotype M26 (35) as controls. In brief, 48-h C. difficile cultures grown on blood agar were anaerobically resuspended in antibiotic-free brain heart infusion broth supplemented with 0.5% yeast extract (Acumedia, Lansing, MI) at an optical density of 0.600 of 0.3. Ten milliliters of those suspensions was placed inside 6-mm-diameter pretreated dialysis tubing (Spectra/Por, 12–14,000 MWCO, Spectrum Laboratories, Rancho Dominguez, CA), which were submerged in 500 mL of brain heart infusion–yeast broth and anaerobically incubated at 37°C for 72 h. At the end of the assay, the broth within the tubing were harvested, centrifuged (10,000 × g for 5 min) and filtered (0.2-μm filters) for sterile testing on 90% confluent monolayer cultures of Vero cells line. Vero cells were grown with Dulbecco's modified Eagle's high glucose medium (GIBCO, Invitrogen, Carlsbad, CA) supplemented with penicillin and streptomycin (100 U and 0.1 mg/mL, respectively; Sigma Aldrich) and 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO), but tested for cytotoxin activity with the medium only (80 μL; Dulbecco's modified Eagle's high glucose medium with antibiotics), 1% fetal bovine serum, and 20 μL of the toxin filtrate. Cytotoxicity (cell rounding) of twofold serial dilutions was quantified 24 h after toxin inoculation, in duplicate, with 96-well microtiter plates (Corning Life Sciences, Corning, NY). A virologist blindly assessed the plates with a scoring system, with determined kappa agreement between ratings greater than 0.95.

**Antimicrobial agent susceptibility.** Commercial E-test strips (AB Biodisk, Solna, Sweden) were used to determine the MICs against six antimicrobial agent classes of clinical relevance in humans: metronidazole and vancomycin (first choice treatments for C. difficile infections [CDI]) (7), moxifloxacin and clindamycin (widely associated with CDI induction) (4, 7), and linezolid and tigecycline (newer therapeutic classes against CDI) (18, 29). Single isolates from seven fed and seven mature cattle representing all PCR ribotypes were tested. In brief, 24-h-old C. difficile colonies grown on blood agar and resuspended (inside anaerobic chamber) in Brucella broth (Oxoid, Ltd.) were used at an OD₆₀₀ of 0.23 ± 0.01 to lawn 6-h prerduced Brucella agar, where the six E-test strips were seeded. Plates were read after 48 h of anaerobic incubation at 37°C, following the manufacturer's recommendations. MICs and interpretive categories for metronidazole, clindamycin, and moxifloxacin were from the Clinical and Laboratory Standards Institute, while the values for vancomycin,
linezolid, and tigecycline were from studies published previously \((16, 18, 22, 34)\).

**Magnitude of fecal shedding.** The quantification of *C. difficile* shedding (log CFU per gram of feces) was determined with the standard spread-plate method. Six available frozen samples that were *C. difficile* positive by the enrichment method and three negative samples were enumerated. In short, duplicate 2-g aliquots of feces homogenized with phosphate-buffered saline (PBS; 1:1 [vol/vol]) were split into two subsamples, one for direct plating and one for ethanol shock. Ethanol was used to inactivate vegetative fecal contaminants to facilitate enumeration of *C. difficile* spores \((2)\). Then, \(10^5\) to \(10^6\) dilutions were spread plated onto blood and *C. difficile* selective agars. After 72 h of incubation, *C. difficile*-like colonies were enumerated, subcultured onto blood agar, and confirmed with multiplex PCR \((26)\) and PCR ribotyping \((3)\). A reference strain of *C. difficile* PCR ribotype 078 was used as control to estimate the detection thresholds of our enrichment and spread-plate methods. For this purpose, duplicates from three samples were spiked with spores of PCR ribotype 078 \((3.4 \text{ log CFU/g})\) that had been aged 4 weeks in PBS.

**Statistics.** Assuming simple random sampling, 120 animals were deemed adequate to test a hypothesized *C. difficile* prevalence of 5.5\% \(\pm\) 6\% for each cattle type at harvest \((\text{one sample, } \alpha = 0.05, \text{power } = 0.8)\). *C. difficile* culture binary data was analyzed with logistic regression with cattle type \((\text{fed versus mature})\) and region as predictors. Fisher’s exact statistics tested proportions with zero counts \((27)\). Confidence intervals of binomials were estimated with exact statistics \((6)\). Nonparametric statistics and significance values for data \((\text{expressed as log CFU per gram})\) were adjusted for ties \((\text{Minitab-15, State College, PA; STAATA-10.1, College Station, TX}). Geometrical means of the MICs \((51)\) were used for descriptive statistics.

**RESULTS**

**Prevalence.** In all, single fecal samples from 944 animals were tested for *C. difficile* \((118 \pm 12\text{ in each processing plant})\). Using the enrichment method, 29 *C. difficile* isolates were recovered from 17 fecal samples \((1.8\%)\), 10 from fed cattle and 7 from mature cattle \((\text{Fig. 1})\). The proportion of *C. difficile* shedders was comparable for all plants and regions, and no differences were observed between fed and mature cattle controlling for region \((\text{odds ratio of shedding in mature cattle } = 0.8; 95\% \text{ confidence interval } [CI] = 0.03, 2.1; P = 0.596)\). Toxigenic *C. difficile* was recovered from 4 \((24\%)\) of the 17 shedding animals; although these 4 animals were all fed cattle, there was no statistical difference when compared with the shedding prevalence in mature cattle \((\text{Fisher’s exact, } P = 0.125)\) \((\text{Table 1})\).

**Genotypes.** PCR ribotyping identified most isolates had unique fingerprints except for a PCR ribotype of tcdA\(^-\), tcdB\(^-\), cdtA\(^-\), cdtB\(^-\) \((\text{toxins A and B not detectable})\) that was common in the western and central regions \((\text{superscripted letter } a\text{ in Fig. 1})\). Dialysis tubing assay confirmed this strain was nontoxigenic \((\text{control ATCC strain cytotoxic titer was } 1:1024)\). Emerging PCR ribotype 078–toxinotype V was identified in the eastern region in one of the four fed cattle shedding toxigenic *C. difficile*. The other three toxigenic isolates were toxinotype 0; each had unique

![FIGURE 1. Clostridium difficile in cattle at harvest](http://meridian.allenpress.com/jfp/article-pdf/74/10/1618/1683865/0362-028x_jfp-11-141.pdf)

**Antimicrobial agent susceptibility.** All isolates tested were susceptible to metronidazole and vancomycin, but many-fold more susceptible to tigecycline \((\text{Fig. 2 and Table 2})\). In contrast, 93\% of isolates had intermediate or resistant MICs for clindamycin. Only one \((7\%)\) isolate was resistant to moxifloxacin. The MICs for linezolid were...
comparable to moxifloxacin and metronidazole but were among the highest values reported for *C. difficile* isolates of human origin (1, 18). One of the toxigenic isolates (toxinotype 0) was multidrug resistant and had the highest measurable MICs for clindamycin and moxifloxacin and among the highest MICs reported for linezolid (Fig. 2). Using the actual MICs, the cutoffs for resistance, and the geometrical means as previously reported (51), *C. difficile* isolates from fed and mature cattle had comparable levels of susceptibility to the drugs tested (P > 0.05). However, *C. difficile* from fed cattle had more variability because of MICs in toxigenic isolates (Bartlett’s test controlling for cattle type and strain toxinotype, P < 0.001; Fig. 2).

**Magnitude of shedding.** Analysis of enumerated plate series where *C. difficile* was confirmed (n = 28) indicated that the median of shedding of *C. difficile* at the time of harvest was 2.2 log CFU/g of feces, but in some cases it reached almost 5 log units (range = 1.6 to 4.8; 95% CI = 1.6, 4.3). When duplicate samples were spiked with spores from strain PCR ribotype 078, our spread plate–serial dilution method recovered a slightly lower number of spores than what was expected (median = 0.45 log CFU/g lower; 95.7% CI = 0.076, 1.283; Mann-Whitney, P = 0.083; Fig. 3). In contrast, direct plating of PBS suspensions of PCR ribotype 078 yielded 1.2 log units more *C. difficile* than matched samples treated with ethanol shock (95% CI = 0.9, 1.5; Mann-Whitney, P = 0.011; Fig. 3).

**DISCUSSION**

This enrichment-based study indicate that the prevalence of fecal shedding of toxigenic *C. difficile* in cattle at harvest (0.4% [4 of 944]) is in agreement with a recent longitudinal study conducted in a naturally infected feedlot (0.6% at harvest) (31), but it is much lower than the prevalence reported in calves at the farm (7 to 13%) and on entry to veal calf operations (33 to 36%) (8). It is, however, important to note that the present study was conducted in summer when *C. difficile* shedding in neonatal calves (and possible the extent and susceptibility to environmental contamination) is the lowest (34).

The toxigenic *C. difficile* isolates here identified correspond to important toxinotypes associated with CDI in humans, toxinotypes 0 and V. PCR ribotype 078–toxinotype V is an emerging *C. difficile* genotype, increasing its participation in human disease and its presence in food animals and foods (19, 38, 41).

![FIGURE 2. MICs of cattle-derived *Clostridium difficile* isolates to six antimicrobial classes of relevance for human *C. difficile* infections (CDI). Susceptibility zones within antibiotic ranges tested are illustrated. Vertical bars indicate published linezolid MIC ranges for human isolates: letter a for reference (1) and b for reference (52).](http://meridian.allenpress.com/jfp/article-pdf/74/10/1618/1683865/0362-028x_jfp-11-141.pdf)
isolation of emerging PCR ribotype 078 from one of our fed cattle in one of the meat processing plants in the eastern region is of epidemiological interest. This genotype has also been isolated from dairy cattle in Ontario, Canada (34), and from finishing steers (31) and farmed deer in Ohio (13), indicating that PCR ribotype 078 might have the potential for geographical distribution across farming operations in the east. Of complementary interest, the most common genotype identified in meat plants of the western and central regions was a nontoxicogenic PCR ribotype (the letter a in Fig. 1A) that has not been reported in our studies on farm animals in the east. Understanding the factors associated with the dissemination of C. difficile in livestock and the shedding prevalence (toxigenic:nontoxicigenic), which can vary along the production system, is important, because nontoxicigenic C. difficile has been proposed as a probiotic strategy to prevent C. difficile colonization in animals and people (39, 40).

The magnitude of C. difficile shedding ranged from about 1 to 5 log CFU/g of feces. This shedding variability could represent actual temporal variation in vivo, local heterogeneity of bacterial concentration within the fecal samples (25), or simply the variability of enumerations methods. In our study, ethanol shock of control strain PCR ribotype 078 spores yielded fewer spores than with direct plating. This indicates that our enumeration of spores in fecal samples is likely an underestimation of the real C. difficile shedding in cattle. Recently, a study of culture methods by Thitaram et al. (44), comparing single versus double alcohol shock treatments as part of an enrichment protocol similar to ours, demonstrated differential performance across food animal production systems, and that single alcohol shock (used in our study) was best to concurrently assess the prevalence of C. difficile in dairy and beef cattle feces (44). Comparing the observed prevalence of C. difficile in both studies for single alcohol shock and plating onto sodium taurocholate–clysosiner–cefoxitin–fructose agar results, there were no differences for mature cattle (7 [1.5%] of 462 versus 19 [1.4%] of 1,325; chi-square, P = 0.9). However, C. difficile was significantly more prevalent in beef cattle in Thitaram’s study (10 [2.1%] of 482 versus 157 [5.3%] of 2,965; chi-square, P = 0.002). Collectively, both studies indicate that fecal shedding of C. difficile in beef feedlot cattle could be more subject to regional or production variability than in mature cattle.

### TABLE 2. Antimicrobial agent susceptibility of Clostridium difficile from cattle at harvesta

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC90</th>
<th>MIC90</th>
<th>Max</th>
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<tr>
<td>Metronidazole</td>
<td>1.0</td>
<td>1.5</td>
<td>1.5</td>
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<tr>
<td>Vancomycin</td>
<td>0.75</td>
<td>0.75</td>
<td>1.5</td>
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<tr>
<td>Linezolid</td>
<td>1.5</td>
<td>2.0</td>
<td>8</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.036</td>
<td>0.064</td>
<td>0.5</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>6</td>
<td>12</td>
<td>256</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>1.0</td>
<td>1.5</td>
<td>32</td>
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a Determined with the E-test method and presented in micrograms per milliliter. MIC90, MIC for 50% of strains tested; MIC90, MIC for 90% of strains tested.

It is uncertain if fecal shedding of large numbers of C. difficile persists over time or if it is an oscillating pattern (5). Periods of fecal shedding of greater than 3 or 4 log units have been referred by some as “supershedding” status, a determinant for infectousness (5). Supershedding has recently been described for C. difficile in response to antimicrobial agent therapy in mice (20), but the factors associated with the magnitude of fecal shedding in cattle and its causal role in food contamination are unknown. The identification of up to 4.8 log CFU/g of feces indicates that small amounts of feces could be sufficient to contaminate meat processing facilities, equipment, beef carcasses, and processed foods.

There is an important contrast between this and other studies conducted at harvest (31), and studies with similar culture methods conducted on retail meat products: The low prevalence of C. difficile shedding at harvest (<1.2%) seems remarkably different from the much higher proportion of C. difficile contamination observed in retail products (6 to 42%) (32, 41). Identifying postharvest factors that could contribute to product contamination and proliferation of C. difficile within foods is important, especially because emerging strains can survive minimum cooking recommendations for ground meats (30, 33).

In contrast to other reports of cattle- and meat-derived C. difficile isolates in which 100% of fluoroquinolone resistance was observed (34, 35), all our isolates, except one, were susceptible to moxifloxacin. The regulations in the United States restrict fluoroquinolone usage in food animals (12, 49, 50). Enrofloacin, the only fluoroquinolone permitted, is restricted to treatment of nonresponsive respiratory disease in (i) beef cattle and (ii) nonlactating replacement dairy heifers younger than 20 months of age. Explicitly, enrofloacin is prohibited for dairy cattle or calves intended for production of beef (47), although noncompliance might occur (49, 50).
Purposely, we chose tigecycline and linezolid, which are newer drug classes, with emerging potential for treatment of CDI, to contribute to the general understanding of susceptibility in *C. difficile*. These drugs exhibit marked inhibitory activity against bacterial strains resistant to other antimicrobial agent classes, including fluoroquinolones. Because these antimicrobial agents are relatively new and not used in cattle in North America, it would be unlikely that resistance in cattle-derived isolates could be attributable to the use of such medications on farms. The isolates recovered in this study displayed the same high susceptibility documented for tigecycline in global surveillance programs (17, 22). However, high MICs of linezolid were required to inhibit most *C. difficile* isolates. The MICs found here are comparable to the highest MICs reported for human-derived isolates (52). Of public health relevance, the isolate that exhibited the highest MIC for linezolid (8 μg/ml) was also highly resistant to clindamycin and moxifloxacin and was toxigenic. Although clindamycin is not approved for use in food animals (48), most isolates had intermediate or resistant MICs to clindamycin.

The source of the linezolid-clindamycin-moxifloxacin resistance in the abovementioned toxigenic *C. difficile* isolate is uncertain. However, since the use of these antimicrobial agents, particularly linezolid, in commercial cattle for harvest is unlikely in North America, it is possible that this resistant strain originated from an environmental source or indirectly from species where those antimicrobial agents are more commonly used, including humans. Transfer of resistance from other enteric bacteria (e.g., *Enterococcus* spp.), where intrinsic resistance might occur (particularly linezolid) (11, 53), is another possibility.

In summary, this study indicates that live cattle might be a source of *C. difficile* contamination of meat processing facilities at the time of harvest, but the prevalence of cattle harboring this pathogen at the time they enter the food chain is low. Of epidemiological concern, two common toxigenic genotypes of *C. difficile* (toxinotype 0 that in addition was linezolid-clindamycin-moxifloxacin resistant, and emerging toxinotype V–PCR ribotype 078, which has been increasingly isolated from animals and foods) were identified in cattle at harvest. Understanding how nontoxigenic *C. difficile* modulate fecal shedding of toxigenic strains in various steps of food production provides insight into prevention strategies to reduce the risk of food contamination.

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