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Effect of Subtherapeutic Administration of Antibiotics on the Prevalence of Antibiotic-Resistant *Escherichia coli* Bacteria in Feedlot Cattle▽

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Antibiotic-resistant *Escherichia coli* in 300 feedlot steers receiving subtherapeutic levels of antibiotics was investigated through the collection of 3,300 fecal samples over a 314-day period. Antibiotics were selected based on the commonality of use in the industry and included chlortetracycline plus sulfamethazine (TET-SUL), chlortetracycline (TET), virginiamycin, monensin, tylosin, or no antibiotic supplementation (control). Steers were initially fed a barley silage-based diet, followed by transition to a barley grain-based diet. Despite not being administered antibiotics prior to arrival at the feedlot, the prevalences of steers shedding TET- and ampicillin (AMP)-resistant *E. coli* were >40 and <30%, respectively. Inclusion of TET-SUL in the diet increased the prevalence of steers shedding TET- and AMP-resistant *E. coli* and the percentage of TET- and AMP-resistant *E. coli* in the total generic *E. coli* population. Irrespective of treatment, the prevalence of steers shedding TET-resistant *E. coli* was higher in animals fed grain-based compared to silage-based diets. All steers shed TET-resistant *E. coli* at least once during the experiment. A total of 7,184 isolates were analyzed for MIC of antibiotics. Across antibiotic treatments, 1,009 (13.9%), 7 (0.1%), and 3,413 (47.1%) *E. coli* isolates were resistant to AMP, gentamicin, or TET, respectively. In addition, 131 (1.8%) and 143 (2.0%) isolates exhibited potential resistance to extended-spectrum β-lactamases, as indicated by either ceftazidime or cefpodoxime resistance. No isolates were resistant to ciprofloxacin. The findings of the present study indicated that subtherapeutic administration of tetracycline in combination with sulfamethazine increased the prevalence of tetracycline- and AMP-resistant *E. coli* in cattle. However, resistance to antibiotics may be related to additional environmental factors such as diet.

In North America, antibiotics have been used in beef cattle production since the 1950s, for therapy, as prophylactics against bacterial infection, and as antimicrobial growth promoters (AGP). Antibiotics used for nontherapeutic applications are generally administered in the diet, either at specific times of high disease risk such as upon arrival at the feedlot or on a continuous basis to improve feed efficiency. As AGP, antibiotics are fed to cattle to improve feed utilization (46) and the efficiency of meat and milk production through alterations in rumen microbial fermentation (5, 37) and metabolism (61). Most cattle in North America receive AGP at some point during production (24, 62).

The use of AGP has the potential to contribute to the emergence of antibiotic-resistant commensal and/or pathogenic bacteria (51). The fact that AGP are administered continuously at low concentrations has been hypothesized to increase the risk of the development of resistance compared to antibiotics that are administered therapeutically (30). Of particular concern are AGP that are used in both human and veterinary medical applications (e.g., tetracycline) or that share a common antibiotic family class (e.g., the macrolide tylosin and the streptogramin virginiamycin) with antibiotics essential for treatment of bacterial diseases in humans. There is strong evidence that antibiotic-resistant bacteria can be transferred from livestock to humans (4, 60) and, consequently, concern for human health, as well as consumer and political pressure, prompted the European Union to ban AGP in 1999 (11).

Study of the effect of AGP on antibiotic resistance has focused mainly on swine and poultry. Despite the fact that over 2 million kilograms of AGP are administered to cattle in North America each year, there are few reports on the effects of feeding AGP on the development of antibiotic resistance during beef cattle production (36). The studies that have examined antibiotic-resistant bacteria in cattle have typically used clinical isolates submitted to veterinary laboratories, an approach that likely overestimates the actual prevalence of antibiotic-resistant bacteria within cattle populations (22). Furthermore, in most of these studies, the history of prior antibiotic use is incomplete, and the nature of the samples analyzed makes it impossible to track the course of antibiotic resistance in a single animal over time.

Escherichia coli accounts for up to 1% of the colonic bacteria in cattle (17). Because of the prominence of *E. coli* in the gut and because it has been shown to account for the majority of

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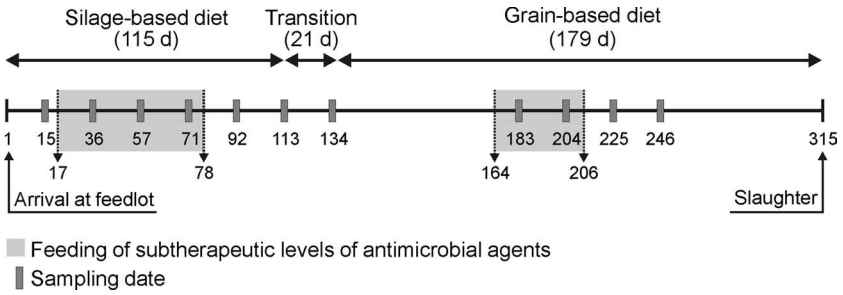

FIG. 1. Schematic representation of the timeline of the experiment. Day numbers are shown within dietary feeding regimen. Animals were fed a silage-based diet and were then transitioned to, and maintained on, a grain-based diet. Gray regions indicate periods during which antibiotics were top dressed onto feed in the trough. Periodic dark rectangles indicate sampling dates, upon which two fecal swabs were obtained from each of the 300 steers.

resistance in *Enterobacteriaceae*, (44), this bacterium has been postulated to serve as a reservoir of antibiotic resistance genes within the digestive tract (23, 28). Moreover, *E. coli* readily exchanges genetic material with other bacterial species (6, 15), and it is possible that this organism may pass antibiotic resistance genes to transient bacterial pathogens that cause disease in humans (27). Thus, *E. coli* is a logical indicator of the extent of antibiotic resistance within microbial populations of the bovine digestive tract.

The antimicrobial feed additives tested in the present study were selected because they are typically used in North American beef production (43). The objectives of the present study were to determine (i) the extent to which subtherapeutic administration of antibiotics (viz., chlortetracycline, chlortetracycline and sulfamethazine in combination, virginiamycin, monensin, or tylosin) affects the proportion of cattle shedding *E. coli* resistant to antibiotics relevant to the treatment of bacterial disease in humans (i.e., tetracycline, ampicillin, gentamicin, and ciprofloxacin) and (ii) to determine whether the prevalence of cattle shedding antibiotic-resistant *E. coli* would decrease upon removal of these additives from the diet.

MATERIALS AND METHODS

Animals and treatments. Three hundred crossbred steers (initial body weight, 198 ± 20 kg) were blocked by weight and then randomly assigned to 30 pens at the Lethbridge Research Centre feedlot. The steers were brought directly to the feedlot from a common range (Deseret Ranches, Raymond, Alberta, Canada) and received no antibiotics prior to the initiation of the experiment. In addition, AGP had not been administered to cattle in the feedlot prior to the present study. Five pens (10 steers per pen) were assigned to each of six treatments: (i) control, no antibiotics; (ii) chlortetracycline and sulfamethazine (each at 44 ppm; fed as Aureo S-700 G; Alpharma, Inc., Bridgewater, NJ; treatment denoted TET-SUL); (iii) chlortetracycline (11 ppm; fed as Aureomycin-100 G; Alpharma; treatment denoted TET); (iv) monensin (25 ppm, fed as Rumensin, Elanco Animal Health, Calgary, Alberta, Canada; treatment denoted MON); (v) tylosin phosphate (11 ppm, fed as Tylan, Elanco Animal Health; treatment denoted TYL); and (vi) virginiamycin (31 ppm, fed as V-Max, Pfizer Animal Health, New York, NY; treatment denoted VIR). Other than virginiamycin, these antibiotics and concentrations were selected on the basis of their commonality of use in the Canadian feedlot industry. Virginiamycin was included in the study because it is not registered for use in Canada and, as a result, neither calves nor their dams would have had prior exposure to this antibiotic. Adjacent pens in the feedlot were supplied by a common watering bowl, but assignment of treatments to pens was arranged so that only cattle in the same treatment group drank from the same bowl.

Steers were fed diets typical of feedlots in Western Canada. For the first 115 days in the feedlot, steers were fed a silage-based, background diet consisting of 70% barley silage, 25% barley grain, and 5% mineral and vitamin supplement (dry matter basis) (Fig. 1). Steers were then adapted to a barley grain-based finishing diet consisting of 85% barley, 10% barley silage, and 5% supplement (dry matter basis) over a 21-day transition period and were then maintained on this diet for an additional 179 days. The present study was approved by the Lethbridge Research Centre Animal Care Committee, with all cattle being cared for according to the guidelines of the Canadian Council on Animal Care (10).

The steers were fed once daily in a manner that ensured that all feed given was consumed each day. To avoid cross-contamination of feed, antibiotics were mixed with 5 kg of supplement and manually spread over the surface of the feed in each trough, immediately after delivery of the feed. The feed troughs were of sufficient length that all steers in a pen could attend at the same time. Steers assigned to the control treatment had no access to medicated feed at any time during the experiment.

Antibiotics were introduced into the diets 17 days after the steers' arrival at the feedlot and were included in the silage-based diet for 61 days. Antimicrobial supplementation was discontinued for 86 days, and then antibiotics were reintroduced for an additional 42-day period during the feeding of the grain-based diet (Fig. 1). The removal of antibiotics was included in the study to determine whether withdrawal could be used as a management practice to reduce the prevalence of antibiotic-resistant *E. coli* should it arise. In addition, in some feedlots, withdrawal of medicated feed is used as a standard practice in growth performance and disease management.

Sample collection and microbiology. Collection of fecal material from each animal was initiated on 11 sampling days (the total sampling time spanned 2 days) during the experiment (Fig. 1). Two clinical swabs (Starswap, Starplex Scientific, Ontario, Canada) per steer were inserted approximately 5 cm into the rectum and rotated until covered with a uniform amount of feces. Swabs were placed individually in sterile, capped test tubes and transported immediately to the laboratory (0.5 km). All samples were processed within 4 h of collection. At the lab, swabs were broken and mixed with 750 μl of brain heart infusion broth (BHI; Difco/Becton Dickinson, Sparks, MD) amended with glycerol (20% [vol/vol]). The tubes containing swabs and medium were vortex mixed thoroughly, and the slurries from both tubes for each steer were combined.

Fecal slurry from each steer was plated onto MacConkey agar (Difco/Becton Dickinson) containing no antibiotics (MAC) or onto MAC amended with ampicillin (50 μg ml^{-1}; MAC+AMP), tetracycline hydrochloride (4 μg ml^{-1}; MAC+TET), gentamicin (2 μg ml^{-1}; MAC+GEN), or ciprofloxacin (2 μg ml^{-1}; MAC+CIP). Aliquots of 10 μl of slurry were plated onto each of the selective media. For plating onto nonselective agar (MAC), 100 μl of slurry was diluted with 2.9 ml of BHI broth, and 50-μl volumes were plated. Plates were incubated for 24 h at 39°C before lactose-positive colonies were counted, and those plates on which no colonies were evident were incubated for an additional 24 h to confirm the absence of bacteria. Colony counts from each of the selective media were compared against counts from nonselective (MAC) plates to estimate percentages of presumed antibiotic resistance by and across sampling days, treatments (antibiotic-supplemented feeding groups), and diet formulations (silage-based versus grain-based).

Concentrations of antibiotics used in the MAC+TET, MAC+GEN, and MAC+CIP plates were deliberately set below the standards for defining resistance described by the National Committee for Clinical Laboratory Standards (NCCLS) (40), now the Clinical and Laboratory Standards Institute (CLSI) (13), to maximize the likelihood of isolating resistant *E. coli*. The concentration of ampicillin selected for the MAC+AMP plates exceeded NCCLS standards (50

versus 32 μg ml^{-1}) but was chosen to prevent the overgrowth of plates from interfering with counting colonies. The antibiotics selected for study were chosen on the basis of commonality of use between cattle and humans, in the case of tetracycline, or their importance in treating human infections, in the case of ampicillin, gentamicin, and ciprofloxacin.

Two colonies were picked from each nonselective plate, and one or two were picked from each of the antibiotic-selective plates. Selected colonies were streaked onto Trypticase soy agar (Difco) and incubated at 39°C for 24 h. Colonies on the Trypticase soy agar plates were transferred to BHI and glycerol and stored at −80°C for further analysis (below). Also, 300 of these isolates (75 each from the MAC, MAC+AMP, MAC+TET, and MAC+GEN plates) were subsampled for confirmation of their identity as *E. coli* on the basis of lactose utilization, methyl red reaction, β-galactosidase and β-glucuronidase activities, and fatty acid methyl ester analysis (18).

Antimicrobial susceptibility testing. Susceptibility to ampicillin (2, 4, 8, 16, and 32 μg ml^{-1}), gentamicin (0.5, 1, 2, 4, 8, and 16 μg ml^{-1}), and tetracycline (1, 2, 4, 8, and 16 μg ml^{-1}), as well as screening for reduced susceptibility to ceftazidime (0.5, 1, and 2 μg ml^{-1}) and cefpodoxime (0.5, 1, and 2 μg ml^{-1}), were analyzed by using the agar dilution method of CLSI (40) on 7,166 *E. coli* isolated from MAC (n = 3,512), MAC+TET (n = 2,205), MAC+AMP (n = 637), and MAC+GEN (n = 812) plates. Interpretations of isolates resistant to ampicillin, gentamicin, and tetracycline were determined by using antibiotic breakpoint concentrations on the basis of NCCLS (41) recommendations. Ceftazidime and cefpodoxime were included to screen for candidate extended-spectrum beta-lactamase (ESBL)-producing strains of *E. coli* (41). The reference strains *E. coli* (ATCC 25922), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213) were included with each susceptibility determination to confirm proper function of the assay.

PFGE. After preliminary examination of prevalence data and shedding patterns, each of the ampicillin-resistant isolates collected from the control steers on days 15, 113, and 246 (80 isolates total) were selected from storage and subtyped by pulsed-field gel electrophoresis (PFGE) separation of XbaI-digested genomic DNA according to the Centers for Disease Control and Prevention (12) 1-day standardized laboratory protocol for molecular subtyping of *E. coli*. *E. coli* E318N (kindly made available by A. Borczyk, Enteric Reference Laboratory, Ministry of Health, Toronto, Ontario, Canada) was included in each PFGE gel as a control.

Definitions and statistical analyses. Bacteria isolated from selective antibiotic plates were categorized as presumed resistant to the respective antibiotic. The percentage of *E. coli* resistant to the antibiotic concentrations in selective plates was calculated for each antibiotic, with consideration for dilutions, as follows: (number of colonies on the antibiotic-supplemented MAC plates/total colonies counted on nonselective MAC) × 100. Bacterial population estimates were expressed as the $\log_{10}$ CFU swab^{-1}. The prevalence of steers shedding presumed resistant *E. coli* from the selective plates was calculated for each sampling day and feeding group as the number of steers from which antibiotic-resistant *E. coli* were isolated (on MAC+antibiotic plates), expressed as a percentage of the total number of steers in that feeding group. Overall, the prevalence was estimated as the percentage of steers from a particular feeding group that were positive for *E. coli* on the antibiotic-containing plates at least once over the course of the entire study.

Data were analyzed by using the "Proc Mixed" procedure of the SAS (52). Because antimicrobial agents were applied to feed provided to the pens of cattle, the pen was considered the experimental unit. The model for analyzing *E. coli* CFU swab^{-1} included the fixed effects of diet (silage- versus grain-based), treatment (antibiotic regimen), and the interaction between diet and treatment. The random effect of a pen nested within treatment was used. The repeated statement was applied to the day of sampling, using the pen nested within treatment as the subject. Various error structures were tested, and the one giving the lowest Akaike information criterion was chosen for analysis. The effect of diet was analyzed by comparing two data sets generated by combining days on which animals consumed a silage-based diet (days 15, 36, 57, 71, 92, and 133) or a grain-based diet (days 134, 183, 204, 225, and 246). Both the silage- and the grain-based data sets contained one sampling point prior to administration of antibiotics (day 15 and day 134, respectively), at least two points from the period during which animals were administered antibiotics (days 36, 57, and 71 and days 183 and 204), and two sampling points that followed withdrawal of antibiotics from the diets (days 92 and 113 and days 225 and 246). For prevalence of resistant *E. coli* isolated from animals, the same statistical model was used, but with the fixed effects of antimicrobial treatment, day, and the interaction of antimicrobial treatment and day. In addition, analysis by diet was conducted. Where appropriate, the LSMEANS function in SAS was used to identify statistical differences.

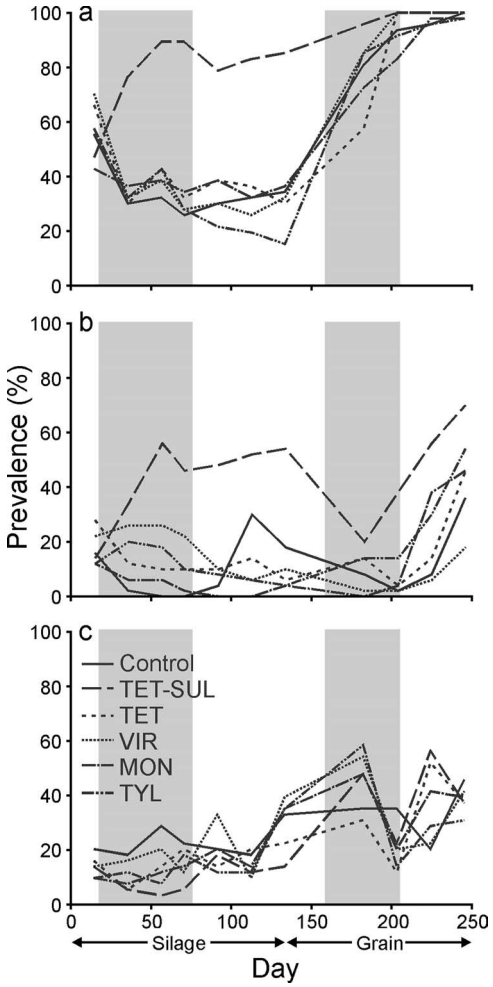

FIG. 2. Prevalence of steers harboring fecal *E. coli* capable of growth on MAC+TET (a), MAC+AMP (b), or MAC+GEN (c) over a 246-day feeding trial during which fecal samples were collected on 11 occasions. Gray areas indicate periods during which antibiotics were supplemented onto feed in the troughs. Line styles distinguish the antibiotics fed (n = 50). The antimicrobial treatments are described fully in Materials and Methods.

RESULTS

Isolation of *E. coli* strains presumed resistant. In total, approximately 3,300 fecal samples were processed for isolation of *E. coli* on plates supplemented with (MAC+AMP, MAC+CIP, MAC+GEN, and MAC+TET) or without (MAC) antibiotics. No isolates were recovered from any of the MAC+CIP plates. Of the 300 isolates tested for *E. coli* confirmation, more than 99% were identified as *E. coli* by biochemical and fatty acid methyl ester analyses (data not shown). The prevalence of isolation and resistant population data were analyzed as *E. coli* resistant to, and capable of growth on, the concentrations of antimicrobials in the selective plates. These *E. coli* strains were presumed resistant to antimicrobial agents in the selective plates.

(i) Prevalence of isolation. On the first sampling day after arrival at the feedlot (day 15), more than 40% of the steers shed tetracycline-resistant *E. coli* (Fig. 2a), whereas fewer than

TABLE 1. Numbers of *E. coli* isolates from pens housing steers fed silage- or grain-based diets containing antimicrobial agents

Parameter	Antimicrobial agent(s) administered[a]						SE	P^b
	Control	TET-SUL	TET	VIR	MON	TYL		
No. of *E. coli* (log CFU swab^{-1}):								
Silage-based diet[c]	5.32 a	5.00 b	5.00 b	5.18 a	5.16 a	5.13 a	0.07	0.0494
Grain-based diet	5.32	5.36	5.24	5.49	5.30	5.37	0.02	0.1755
Overall	5.32	5.13	5.09	5.29	5.21	5.22		
SE	0.02	0.067	0.05	0.05	0.066	0.03		
Silage vs grain[d]	0.80	0.02	0.038	0.01	0.212	0.01		

[a] Control, no antimicrobial agents added to the diet. The TET-SUL, TET, VIR, and TYL treatments are described in detail in Materials and Methods.
[b] Effect of administration of antimicrobial agents.
[c] Within a row, values followed by different letters differ.
[d] That is, the effect (*P* value) of the diet formulation within an antimicrobial feeding group.

30% were shedding ampicillin- or gentamicin-resistant *E. coli* (Fig. 2b and c). With the exception of the TET-SUL feeding group, including antibiotics in the silage-based diet did not increase the prevalence of steers shedding tetracycline-resistant *E. coli*; in fact, the prevalence was lower ($P = 0.05$) on day 36, when diets other than the control treatment were supplemented with antibiotics, than on day 15, when no antibiotics were fed (Fig. 2a). In contrast, feeding TET-SUL resulted in a dramatic increase ($P = 0.001$) in the prevalence of tetracycline-resistant *E. coli* shedders. The prevalence of steers shedding tetracycline-resistant *E. coli* decreased slightly ($P = 0.08$) upon removal of TET-SUL from the silage-based diet (days 92 and 113). However, the high level of shedders from the TET-SUL treatment was generally sustained throughout the experiment. Withdrawing the other antibiotics did not affect the proportion of steers shedding tetracycline-resistant *E. coli* either on silage-based or on grain-based diets. Although the number of animals shedding tetracycline-resistant *E. coli* increased beyond levels recorded for day 134, when animals were fed grain-based diets without antibiotics, this effect was not attributable to antibiotic supplementation (days 164 to 206) during this feeding period (Fig. 2a). When animals were fed the grain-based diets, the prevalence of animals shedding resistant *E. coli* for animals treated with antibiotics was similar to that of the control group, with the exception of the TET-SUL treatment group. Furthermore, in comparing prevalences when animals were fed the silage- versus grain-based diets, the number of steers shedding tetracycline-resistant *E. coli* increased ($P = 0.04$) upon transition to the grain-based diet.

Including TET-SUL in the diet also increased ($P = 0.02$) the prevalence of steers shedding ampicillin-resistant *E. coli* throughout the trial (Fig. 2b). In general, prevalence of steers shedding ampicillin-resistant *E. coli* remained steady or tended to decline during the feeding period until the point that antibiotics were removed from the grain-based diet (days 225 and 246). After the withdrawal of antibiotics from the grain-based diet, the prevalence of steers shedding ampicillin-resistant *E. coli* increased ($P = 0.04$) in all treatment groups. A notable exception to this trend was observed on day 113, when the prevalence of shedding of ampicillin-resistant *E. coli* in the control group was dramatically increased over the day 92 level. The type of diet fed (silage- versus grain-based) had no effect on the prevalence of animals shedding ampicillin-resistant *E. coli*. The prevalence of cattle shedding gentamicin-resistant *E. coli* was not affected by the type of antibiotic fed or by the withdrawal of antibiotics from the diet, but it tended ($P = 0.09$) to be higher with the grain-based diet than with the silage-based diet (Fig. 2c).

Overall, the prevalence of animals shedding resistant *E. coli* at least once throughout the experiment was similar across treatments. *E. coli* strains resistant to 4 μg tetracycline ml^{-1} were isolated from all cattle within each treatment at least once during the study. Observations of shedding of gentamicin-resistant (2 μg ml^{-1}) *E. coli* were similar, with more than 90% of steers shedding these organisms at least once, irrespective of the presence of antibiotics in the diet. Over the course of the 11 sampling times, *E. coli* strains resistant to ampicillin at 50 μg ml^{-1} were isolated from 70% of the steers in the study.

(ii) Antibiotic-resistant populations. With the exception of the control ($P = 0.80$) and MON ($P = 0.212$) treatments, numbers of *E. coli* isolated on nonselective MAC plates from cattle were lower ($P = 0.02$) when the silage-based diet was fed, compared to the grain-based diet (Table 1). Supplementing the silage-based diet with TET ($P = 0.024$) and TET-SUL ($P = 0.01$) lowered the number of *E. coli* isolated in comparison to the control group (data not shown). Variability across treatments in the numbers of *E. coli* isolated was less pronounced when the grain-based diet was fed, but isolate numbers were numerically greater when tylosin or virginiamycin were administered, compared to the control.

In general, *E. coli* isolates culturable on antibiotic-supplemented MAC agar were more numerous when antibiotics were added to the grain-based diet than when they were added to the silage-based diet (Table 2). Changing from the silage- to the grain-based diet tended to increase ($P = 0.09$) the proportion of tetracycline resistance among isolated *E. coli* (MAC+TET plates) irrespective of the dietary antibiotic treatment. The TET-SUL treatment increased the percentage of *E. coli* resistant to tetracycline, compared to the control, for both silage-based ($P = 0.001$) and grain-based ($P = 0.02$) diets. The percentage of ampicillin resistance among the *E. coli* isolates was also increased by the inclusion of TET-SUL in both the silage-based ($P = 0.001$) and grain-based ($P = 0.03$) diets. The degree of resistance to ampicillin (at 50 μg ml^{-1}) among isolated *E. coli* was unaffected ($P = 0.15$) by the concentration of grain in the diet.

The overall percentages of *E. coli* resistant to antibiotic-supplemented MAC plates, across all sampling dates, are

TABLE 2. Percentages of the *E. coli* isolates collected from pens of steers fed antimicrobial agents with silage- or grain-based diets and that were capable of growth on selective media containing antibiotics[a]

Selective medium and parameter	% of *E. coli* isolates from antimicrobial treatment group[b]:						SE	*P*[c]
	Control	TET-SUL	TET	VIR	MON	TYL		
MAC+TET								
Silage-based diet	1.4 b	15.8 a	4.1 b	1.6 b	3.5 b	1.7 b	0.7	0.0011
Grain-based diet	11.6 bc	25.9 a	20.3 ab	5.2 c	9.4 bc	12.4 bc	2.5	0.0001
SE	3.72	4.50	3.37	1.49	2.15	1.30		
Silage vs grain[d]	0.055	0.087	0.008	0.07	0.05	0.001		
MAC+AMP								
Silage-based diet	0.23 b	4.14 a	0.43 b	0.19 b	0.03 b	0.58 b	0.69	0.004
Grain-based diet	0.28 b	0.94 a	0.13 b	0.01 b	0.28 b	0.80 b	0.27	0.145
SE	0.38	1.64	0.42	0.625	0.235	0.39		
Silage vs grain	0.89	0.122	0.51	0.28	0.35	0.60		
MAC+GEN								
Silage-based diet	0.004 ab	0.003 b	0.011 a	0.003 b	0.004 ab	0.002 b	0.002	0.018
Grain-based diet	0.023	0.007	0.011	0.006	0.006	0.005	0.004	0.149
SE	0.01	0.001	0.008	0.001	NE[e]	0.001		
Silage vs grain	0.164	0.148	0.987	0.27	NE	0.08		

[a] Resistance was determined by plating onto MAC+TET, MAC+AMP, or MAC+GEN as defined in the text. Percentages were calculated as follows: (number of colonies on the antibiotic-supplemented MAC plates)/(total number of colonies counted on nonselective MAC). The *E. coli* isolate numbers on MAC plates are indicated in Table 1.

[b] Control, no added antimicrobial agents; the TET-SUL, TET, VIR, MON, and TYL treatments are described in detail in Materials and Methods.

[c] Within a row, values followed by different letters differ.

[d] That is, the effect of diet formulation (*P*).

[e] NE, not executable.

shown in Fig. 3. A significant proportion (>3%) of the *E. coli* population isolated from any treatment group (including the control) exhibited resistance to tetracycline. Including tetracycline in the diet increased the degree of tetracycline resistance in the *E. coli* population to 10% when tetracycline was fed alone (TET treatment; $P = 0.041$), and to 19.5% when it was fed in combination with sulfamethazine (TET-SUL treatment; $P = 0.001$). The TET-SUL treatment also resulted in increased ($P = 0.05$) proportions of ampicillin-resistant strains among the *E. coli* isolates. This was not observed with the TET treatment, which contained no sulfamethazine and lower tetracycline concentrations compared to the TET-SUL treatment (11 ppm versus 44 ppm, respectively). In all cases, less than 0.5% of the *E. coli* population exhibited resistance to gentamicin.

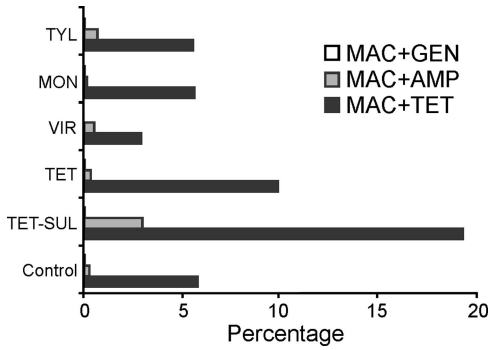

FIG. 3. The overall percentages of bovine fecal *E. coli* isolates that were capable of growth on MAC+GEN, MAC+AMP, or MAC+TET across all sampling dates. Percentages are based on total *E. coli* from nonselective MacConkey agar. Cattle were fed diets supplemented with (TYL, MON, VIR, TET, and TET-SUL) or without (control) antibiotics as described in Materials and Methods.

Antibiotic susceptibility. A total of 7,166 *E. coli* isolates collected from the steers over the course of the study from MAC ($n = 3,512$), MAC+TET ($n = 2,205$), MAC+AMP ($n = 637$), and MAC+GEN ($n = 812$) plates were tested for susceptibilities. The MICs of ampicillin, ceftazidime, cefpodoxime, gentamicin, and tetracycline were determined for each isolate. Isolates were considered resistant if they were capable of growth at or above the resistance breakpoints determined by the CLSI.

(i) Antibiotic MICs for *E. coli* isolates. The MICs of antibiotics for all isolates across antimicrobial treatments are reported in Table 3. The number of resistant *E. coli* strains was variable, depending on the selective media used for isolation. As expected, given that the ampicillin concentration in the MAC+AMP plates exceeded the NCCLS MIC standard of 32 $\mu g\ ml^{-1}$, breakpoint ampicillin resistance was confirmed in virtually all (635 of 637) of the isolates cultured on MAC+AMP. Similarly, a high proportion (2,183 of 2,205) of *E. coli* strains resistant to tetracycline were isolated from plates containing the same selective antibiotic (MAC+TET plates). There appeared to be a genetic linkage between determinants conferring resistance to tetracycline and ampicillin. Of the *E. coli* isolated from MAC+TET plates, 11.4% were resistant to ampicillin compared to 2.2% of those isolated from MAC plates. Conversely, a greater percentage of *E. coli* isolated from MAC+AMP plates were resistant to tetracycline compared to those from the MAC plates (77.7% versus 15.1%).

Isolates exhibiting resistance to gentamicin were recovered only from MAC+GEN plates, and at a low rate. Of the 812 isolates tested from MAC+GEN plates, which contained 2 μg of gentamicin ml^{-1}, only 7 were confirmed as gentamicin resistant. However, of the MAC+GEN isolates, 5.4% were resistant to ampicillin, and 25.1% were resistant to tetracycline.

TABLE 3. Susceptibilities to ampicillin, ceftazidime, cefpodoxime, gentamicin, and tetracycline observed in *E. coli* isolates recovered from feedlot cattle, pooled across subtherapeutic antimicrobial treatment groups

Antibiotic and isolation medium	No. of isolates (%) at MIC[a]:							Total no. of isolates
	0.5 μg ml^{-1}	1 μg ml^{-1}	2 μg ml^{-1}	4 μg ml^{-1}	8 μg ml^{-1}	16 μg ml^{-1}	32 μg ml^{-1}	
Ampicillin								
MAC			0 (0)	3,014 (85.8)	411 (11.7)	8 (0.2)	79* (2.2)	3,512
MAC+TET			0 (0)	1,391 (63.1)	555 (25.2)	8 (0.4)	251* (11.4)	2,205
MAC+AMP			2 (0.3)	-	-	0 (0)	635* (99.7)	637
MAC+GEN			159 (19.6)	538 (66.3)	71 (8.7)	0 (0)	44* (5.4)	812
Subtotal			161 (2.2)	4,943 (69.0)	1,037 (14.5)	16 (0.2)	1,009* (14.1)	7,166
Ceftazidime								
MAC	3,486 (99.3)	3 (0.1)	23* (0.7)					3,512
MAC+TET	2,203 (99.9)	1 (0.1)	1* (0.1)					2,205
MAC+AMP	545 (85.6)	1 (0.2)	91* (14.3)					637
MAC+GEN	792 (97.5)	4 (0.5)	16* (2.0)					812
Subtotal	7,026 (98.0)	9 (0.2)	131* (1.8)					7,166
Cefpodoxime								
MAC	3,441 (98.0)	49 (1.4)	22* (0.6)					3,512
MAC+TET	2,096 (95.1)	106 (4.8)	3* (0.1)					2,205
MAC+AMP	432 (67.8)	102 (16.0)	103* (16.2)					637
MAC+GEN	763 (94.0)	34 (4.2)	15* (1.8)					812
Subtotal	6,732 (94.0)	291 (4.1)	143* (2.0)					7,166
Gentamicin								
MAC		3,480 (99.1)	30 (0.8)	2 (0.1)	-	-		3,512
MAC+TET		2,107 (95.6)	95 (4.3)	3 (0.1)	-	-		2,205
MAC+AMP	246 (38.6)	363 (57.0)	28 (4.4)	0 (0)	0 (0)	-		637
MAC+GEN	209 (25.7)	233 (28.7)	97 (11.9)	200 (24.6)	66 (8.1)	7* (0.9)		812
Subtotal	455 (6.3)	6,183 (86.3)	250 (3.5)	205 (2.9)	66 (0.9)	7* (0.1)		7,166
Tetracycline								
MAC				2,981 (84.9)	0 (0)	531* (15.1)		3,512
MAC+TET				19 (0.9)	3 (0.1)	2,183* (99.0)		2,205
MAC+AMP		0 (0)	59 (9.3)	83 (13.0)	0 (0)	495* (77.7)		637
MAC+GEN		75 (9.2)	490 (60.3)	35 (4.3)	8 (1.0)	204* (25.1)		812
Subtotal		75 (1.0)	549 (7.7)	3,118 (43.5)	11 (0.2)	3,413* (47.6)		7,166

[a] For each antibiotic, the MIC columns in which isolate numbers are marked with asterisks are the breakpoint values (≥) distinguishing sensitive and resistant isolates of *E. coli*, with the exception of ceftazidime and cefpodoxime, for which the indicated concentration was used to screen for candidate *E. coli* potentially encoding ESBLs. Data were analyzed across all of the antibiotic treatments (including control, no antibiotics) described in Materials and Methods. Isolates were recovered from MacConkey agar containing no antibiotics (MAC) or onto MAC amended with ampicillin (MAC+AMP), tetracycline hydrochloride (MAC+TET), or gentamicin (MAC+GEN) as defined in the text. Dashes indicate that isolates were not tested at the specified antibiotic concentration.

From the NCCLS screening procedures, 131 and 142 candidate ESBL-producing *E. coli* isolates were identified by growth on plates containing 2 μg of ceftazidime or cefpodoxime ml^{-1}. With the exception of the MAC+AMP plates, the type of selective medium used to isolate *E. coli* did not affect those with reduced susceptibility to ceftazidime or cefpodoxime. For *E. coli* isolated from MAC+AMP plates, 14.3 and 16.2% of the isolates were capable of growth on plates containing 2 μg ceftazidime ml^{-1} and 2 μg cefpodoxime ml^{-1}, respectively. This accounted for the majority of total candidate ESBL-producing *E. coli* with reduced susceptibility to ceftazidime (91 of 131 isolates) and cefpodoxime (103 of 143 isolates).

(ii) Breakpoint resistance within diet treatments. With the exception of the TET-SUL treatment, the administration of antibiotics to cattle did not affect the development of resistance in *E. coli* to ampicillin, tetracycline, or gentamicin compared to the control group (Table 4). Administration of TET-SUL to the steers increased ($P = 0.06$) the recovery of ampicillin-resistant *E. coli* on MAC, MAC+TET, and MAC+AMP plates. Similarly, the recovery of isolates resistant to tetracycline was higher for the TET-SUL group versus all of the other treatments ($P = 0.01$), when *E. coli* was isolated on MAC, MAC+AMP, or MAC+GEN media. Similar rates of tetracycline resistance (97.4 to 99.7%) were observed among isolates from all treatments, when isolated on MAC+TET plates. *E. coli* strains resistant to gentamicin were recovered from steers in the control, TET-SUL, TET, and VIR treatments. These isolates were only recovered on MAC+GEN plates, and at equally low rates (0.6 to 2.2%) across these treatments. The prevalence of candidate ESBL-producing isolates was evenly distributed among samples from steers in all feeding groups except the MON treatment (data not shown).

Isolates exhibiting coresistance to both ampicillin and tetracycline were isolated from steers within each of the antimicrobial treatments (Table 4). Recovery of tetracycline and ampicillin coresistance was greatest from the TET-SUL treatment. Almost all of the isolates from the TET-SUL treatment that displayed ampicillin resistance were also resistant to tetracycline, regardless of isolation media (83.3 to 100%). Across all antimicrobial treatments, every *E. coli* isolate selected from

TABLE 4. Resistance and coresistance of fecal *E. coli* isolates from steers fed subtherapeutic levels of antibiotics as determined according to the NCCLS standard MIC of antibiotics for human use

Isolation medium[b]	Dietary group[c]	No. of isolates	No. of isolates (%)[a] exhibiting breakpoint resistance to:						
			AMP	TET	GEN	AMP+TET	AMP+GENT	TET+GENT	AMP+TET+GENT
MAC	Control	695	6 (0.9)	36 (5.2)	-	2 (0.3)	-	-	-
	TET-SUL	683	53 (7.8)	284 (41.6)	-	50 (7.3)	-	-	-
	TET	670	4 (0.6)	99 (14.8)	-	1 (0.1)	-	-	-
	VIR	455	6 (1.3)	21 (4.6)	-		-	-	-
	MON	457		38 (8.3)	-		-	-	-
	TYL	552	7 (1.3)	53 (9.6)	-	5 (0.9)	-	-	-
	Subtotal	3,512	76 (2.2)	531 (15.1)	-	58 (1.7)	-	-	-
MAC+TET	Control	332	33 (9.9)	328 (98.8)	-	33 (9.9)	-	-	-
	TET-SUL	503	101 (20.1)	499 (99.2)	-	101 (20.1)	-	-	-
	TET	376	15 (4.0)	373 (99.2)	-	15 (4.0)	-	-	-
	VIR	354	35 (9.9)	352 (99.4)	-	35 (9.9)	-	-	-
	MON	330	20 (6.1)	329 (99.7)	-	20 (6.1)	-	-	-
	TYL	310	47 (15.2)	302 (97.4)	-	47 (15.2)	-	-	-
	Subtotal	2,205	249 (11.3)	2,188 (99.2)	-	249 (11.3)	-	-	-
MAC+AMP	Control	63	62 (98.4)	45 (71.4)	-	45 (71.4)	-	-	-
	TET-SUL	252	252 (100)	240 (95.2)	-	240 (95.2)	-	-	-
	TET	86	85 (98.8)	56 (65.1)	-	56 (65.1)	-	-	-
	VIR	78	78 (100)	47 (60.3)	-	47 (60.3)	-	-	-
	MON	63	63 (100)	36 (57.1)	-	36 (57.1)	-	-	-
	TYL	95	95 (100)	71 (74.7)	-	71 (74.7)	-	-	-
	Subtotal	637	635 (99.7)	495 (77.7)	-	495 (77.7)	-	-	-
MAC+GEN	Control	160	12 (7.5)	23 (14.4)	1 (0.6)	8 (5.0)	-	1 (0.6)	-
	TET-SUL	125	18 (14.4)	87 (69.6)	2 (1.6)	15 (12.0)	-		-
	TET	134	4 (3.0)	29 (21.6)	3 (2.2)	3 (2.2)	2 (1.5)	2 (1.5)	2 (1.5)
	VIR	140	5 (3.6)	19 (13.6)	1 (0.7)	3 (2.1)	1 (0.7)	1 (0.7)	1 (0.7)
	MON	133	3 (2.3)	23 (17.3)	-	1 (0.8)	-	-	-
	TYL	120	2 (1.7)	22 (18.3)	-	2 (1.7)	-	-	-
	Subtotal	812	44 (5.4)	203 (25.0)	7 (0.9)	32 (3.9)	3 (0.4)	4 (0.5)	3 (0.4)

[a] The values shown are the numbers of isolates exhibiting breakpoint resistance to ampicillin (AMP), tetracycline (TET), and gentamicin (GEN) at 32, 16, and 16 μg ml^{-1}, respectively. The numbers of isolates showing resistance to antibiotics are not cumulative. Dashes indicate that no isolates were resistant.

[b] Isolates were recovered from MacConkey agar containing no antibiotics (MAC) or MAC amended with ampicillin (MAC+AMP), tetracycline hydrochloride (MAC+TET), or gentamicin (MAC+GEN).

[c] Control, no antimicrobial agents added to the diet; the TET-SUL,TET, VIR, MON, and TYL antimicrobial feeding groups are defined in Materials and Methods.

MAC+TET plates that was resistant to ampicillin was also resistant to tetracycline. In addition, a high proportion (57.1 to 95.2%) of *E. coli* isolates selected from MAC+AMP plates showed resistance to tetracycline. These data suggested that in many of the isolates, determinants encoding ampicillin resistance were linked to determinants encoding tetracycline resistance. One isolate from the control treatment was resistant both to tetracycline and to gentamicin. Two isolates from the TET and one from the VIR treatments showed coresistance to ampicillin, tetracycline, and gentamicin.

PFGE patterns. Pulsed-field gel electrophoresis of ampicillin-resistant *E. coli* isolated from the control steers on days 15, 113, and 246 provided further insight into the observations of ampicillin resistance. The majority of steers shedding ampicillin-resistant *E. coli* on days 113 and 246 were from pens 19 and 21, respectively (Fig. 4). These groups of animals accounted in large part for the increase in prevalence of steers shedding ampicillin-resistant *E. coli* on those days (Fig. 2). The PFGE patterns of the ampicillin-resistant isolates collected on day 113 from each of the 10 steers in pen 19 were identical to one another (representative data in Fig. 5A) and to those obtained from the four steers in adjacent pen 20 on the same day (data not shown). Similarly, the PFGE patterns of ampicillin-resistant *E. coli* isolates from each of the 10 animals in pen 21 on day 246 were identical (Fig. 5B). Of note, however, is that the patterns produced by pen 19 isolates from day 113 or by the pen 21 isolates from day 246 were not the same as the patterns observed for ampicillin-resistant isolates collected from these same steers (one only in pen 21) on day 15 of the experiment (Fig. 5C and D). The dissimilar PFGE patterns between pens 19 and 21 illustrated that the ampicillin-resistant *E. coli* isolated from these pens were likely different strains.

DISCUSSION

To our knowledge, the present study is the first to examine the impact of administering and of withdrawing dietary antimicrobial growth promoters for intensively reared feedlot cattle on the occurrence and characteristics of antimicrobial-resistant *E. coli* harbored by cattle. Cattle fed two types of diets were administered one of six antimicrobial treatments, and resistant *E. coli* were isolated from selective plates. The use of MacConkey media supplemented with antimicrobial agents to isolate resistant *E. coli* has been described previously (26, 53). In the present study, the prevalence of isolation and resistant microbial population data were based on *E. coli* counts from

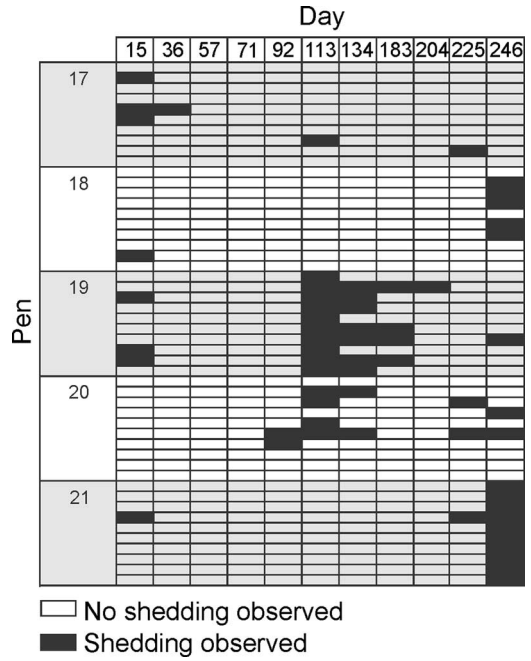

FIG. 4. Schematic representation of detection of ampicillin-resistant *E. coli* in fecal samples from the 50 steers in the control group (no antibiotics fed). The steers were housed in five adjacent pens (17 to 21). Each row represents one of the 10 steers housed in each pen. Column headings indicate sampling days. Filled boxes (black) indicate the days on which fecal isolates resistant to 50 μg of ampicillin ml^{-1} were collected.

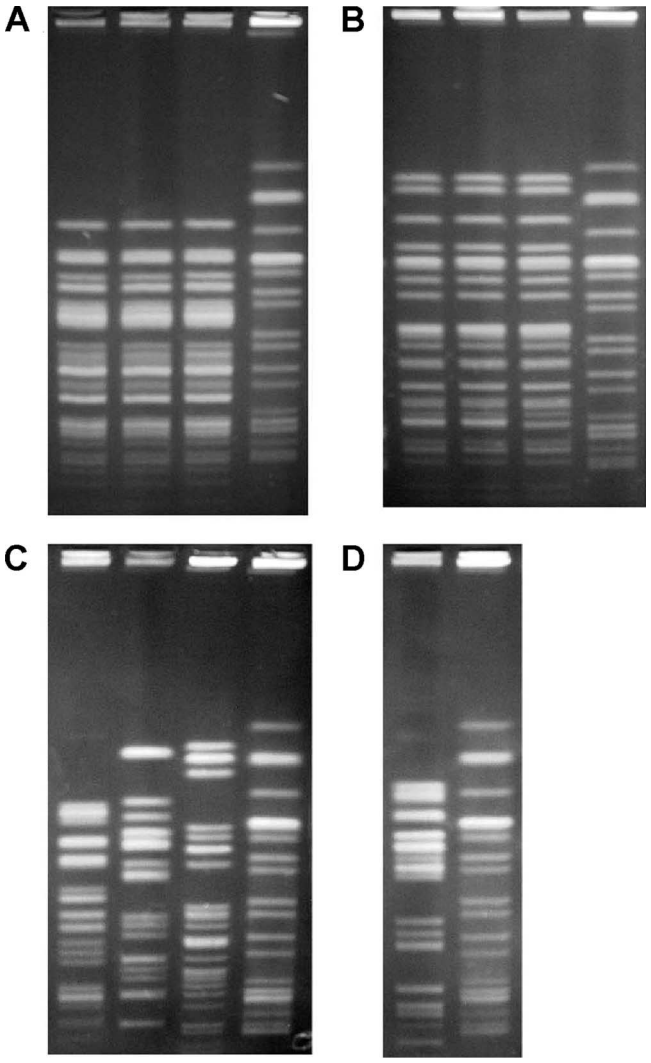

FIG. 5. PFGE of ampicillin-resistant *E. coli* isolates obtained from feces of steers in the control group, i.e., receiving no dietary antibiotic in their feedlot diets throughout the study. In each frame, the lane on the far right contains a reference strain, *E. coli* E318N. Isolates shown in panels A and B were obtained on days on which all 10 steers in a given pen were positive for ampicillin-resistant *E. coli* (see Fig. 4); those in panels C and D were collected on the first sampling day after arrival at the feedlot. (A) Isolates collected on day 113 from three steers in pen 19; (B) isolates collected on day 246 from three steers in pen 21; (C) isolates collected from the same three steers as shown in panel A, but on day 15; (D) isolate from the single steer in pen 21 that was positive for ampicillin-resistant *E. coli* on day 15. Panels A and B contain representative data, since the isolates collected from all 10 steers in these pens produced identical banding patterns. In contrast, the day 15 isolates differed among steers within the pen (in the case of pen 19). Note that within a pen, the banding patterns differed between day 15 isolates and those collected from the same steers later in the feeding period (panel C versus panel A; panel D versus panel B).

selective media that contained concentrations of antibiotics below breakpoint resistance (MAC+TET, MAC+GEN, and MAC+CIP), with the exception of MAC+AMP plates. It was necessary to increase the levels of ampicillin in the MAC+AMP plates beyond the breakpoint for resistance in order to reduce plate overgrowth. Despite this, presumed tetracycline and ampicillin resistance analyzed from colony counts on MAC+TET or MAC+AMP appeared to be a good indication of actual resistance levels. The MICs of tetracycline or ampicillin on *E. coli* from the respective plates indicated that almost all isolates were resistant. In contrast, only 7 of the 813 isolates from the MAC+GEN plates proved resistant to gentamicin as defined by the NCCLS (16 μg ml^{-1}). Thus, the prevalence of gentamicin-resistant *E. coli* in feedlot cattle calculated on the basis of recommended breakpoint would be lower than that depicted in Fig. 2. However, only the differences in tetracycline and ampicillin resistance were attributable to antimicrobial supplementation of diets fed to steers and only for the TET-SUL treatment. This was supported both by the counts of resistant *E. coli* on selective media and by the MIC data.

Including chlortetracycline and sulfamethazine in combination (TET-SUL treatment) in grain-based diets clearly increased the prevalence both of tetracycline-resistant and of ampicillin-resistant *E. coli* in feces from feedlot cattle. Given that the product used in the TET-SUL treatment consisted of a mixture of chlortetracycline and sulfamethazine, it was not possible to determine whether one or both antimicrobial agents were responsible for the increased prevalence of resistance observed in the steers receiving it. We did not control the levels of tetracycline in the treatments, in order to investigate the antimicrobial growth promoters commonly used in the Canadian feedlot industry, each at its recommended concentration. The fact that including chlortetracycline only (TET treatment) in the silage-based diet did not increase the prev-

alence of antibiotic resistance suggested that sulfamethazine may have promoted ampicillin resistance. However, the concentration of chlortetracycline administered as the TET treatment also did not increase the prevalence of tetracycline resistance. Thus, it is possible that 11 ppm was below the selective concentration window required to confer tetracycline resistance (42). Others have proposed that similar concentrations of tetracycline, as applied in the TET treatment, should not select for tetracycline-resistant *E. coli* (49). In contrast, the concentration of chlortetracycline in the diets supplemented with TET-SUL may have been sufficient to confer tetracycline resistance and promote ampicillin resistance as well. This would be especially likely if these determinants shared a common mobile genetic element. Studies have shown that orally administered tetracycline reduced bacterial susceptibility to ampicillin in the cecal flora of poultry (35) and for *E. coli* in swine (20).

There seemed to be a link between tetracycline- and ampicillin resistance determinants in our study. Although the TET-SUL treatment resulted in greater percentages of *E. coli* coresistant to tetracycline and ampicillin, across all treatments there was a strong correlation between coresistance of these two antibiotics. This could be seen particularly among isolates selected from MAC+AMP plates, in which the majority (57.1 to 95.2%) of ampicillin-resistant *E. coli* isolates also exhibited resistance to tetracycline (Table 4). Similar findings have been reported previously, although the extent of coresistance may have been related to the type and age of animal. Sawant et al. (53) described coresistance to at least ampicillin and tetracycline in 42% of *E. coli* isolated from dairy cattle. Coresistance to ampicillin and tetracycline in *E. coli* isolated from newborn calves over a 21-week period was described for 80% of the isolates (25). Studies are currently under way in our laboratory to determine the molecular nature of these resistance determinants. Including dietary antibiotics (i.e., virginiamycin, monensin, and tylosin) targeted primarily against gram-positive bacteria (43, 48) did not alter the prevalence of tetracycline-, ampicillin- or gentamicin-resistant *E. coli*. Interestingly, in another study investigating the use of growth promoters in feedlot cattle, the macrolide tylosin had no effect on erythromycin resistance in *Campylobacter hyointestinalis*; however, tetracycline in the diet of the animals did (29). In the present study, gentamicin resistance was limited, and ciprofloxacin-resistant isolates were not recovered despite the use of selective media. These results are likely due to the infrequent subtherapeutic use of aminoglycosides and Canadian regulatory restrictions against the subtherapeutic use of fluoroquinolones in beef cattle. Thus, the data presented here provide an important baseline of resistance for these antimicrobials should they become more widely used in the industry.

Although the cefpodoxime susceptibility concentration for identifying candidate ESBL-producing *E. coli* has been increased to 4 $\mu g\ ml^{-1}$ (13) in the interim since the work in the present study was conducted, the majority of isolates suspected as ESBL producers on the basis of cefpodoxime MIC were the same isolates identified by the ceftazidime MIC. The observation that candidate ESBL-producing *E. coli* isolates were collected during the silage-based as well as the grain-based feeding phases, and from all treatment groups except MON, makes it difficult to understand the environmental triggers for these potential pathogens. Coselection of CMY-2-producing *E. coli* by treating animals with non-beta-lactams has been reported, and the same has been suggested to be possible for CTX-M-1- and CTX-M-14-producing strains (7). This appeared not to be the case for the subtherapeutic antimicrobials used in the present study, in which most of these resistant *E. coli* strains were isolated from MAC+AMP plates. The low overall numbers of *E. coli* isolates with reduced susceptibility to ceftazidime and cefpodoxime (1.8 and 2.0%, respectively) were similar to levels of resistance reported previously for *E. coli* isolates from herds of dairy calves and cows, which ranged between 0 and 3.5% (16). Although isolates in that study were not subjected to molecular characterization, CTX-M-1- and CTX-M-15-type beta-lactamases have been identified in *E. coli* from food animals (cattle, swine, and poultry) in France (38). Although we only screened for candidate ESBL-producing *E. coli*, considering the clinical importance of ESBL-positive *E. coli* (59) and the fact that cattle have been confirmed as the source of infection of a child with a ceftriaxone-resistant *Salmonella* strain (19), it is clear that further study is necessary to determine the role of cattle in disseminating ESBL-producing *E. coli*.

Despite their having had no previous exposure to tetracycline, the proportion of feedlot steers harboring tetracycline-resistant *E. coli* strains exceeded 40% even before antimicrobial agents were included in the diet. Similarly, ampicillin-resistant isolates were obtained from ca. 10 to 20% of the steers within each treatment before feeding antimicrobials. These results demonstrate that tetracycline- and ampicillin-resistant *E. coli* were harbored by a large number of cattle shortly after arrival at the feedlot, independent of any direct exposure to antibiotics. Presumably, the majority of these resistant *E. coli* would have been acquired from the range environment in which the calves were raised, either from their dams or other environmental sources. Survival of *E. coli* has been shown to occur up to 150 days in fecal pats on range land (57), and stored feed has also been implicated as a source of antimicrobial-resistant *E. coli* at feedlots (14). These sources of resistant bacteria may have been a factor in the colonization of gastrointestinal tracts of calves in our study.

On days 113 and 246, the prevalence of shedding ampicillin-resistant *E. coli* within the control treatment increased substantially. The increase resulted from animals within select pens only, and therefore we chose to analyze the genetic relatedness of the resistant isolates from these animals. The differences observed between PFGE profiles produced by the ampicillin-resistant isolates obtained in pens 19 and 21 early in the study (day 15) and those arising from isolates collected later (day 113 in pen 19; day 246 in pen 21) suggested that the resistant isolates present initially were not those responsible for the large increase in prevalence of ampicillin resistance in control cattle recorded on the latter sampling days in these two pens (Fig. 4). These strains may have been derived from environmental sources. Temporal colonization of calves with select *E. coli* strains from the environment has been described elsewhere (25). Whereas the PFGE profiles for isolates from pen 19 (day 113) and pen 21 (day 246) were distinct, the profiles of isolates from within a pen were identical among all 10 steers. Conservation of strains within pens likely indicated that clonal ampicillin-resistant *E. coli* disseminated readily among pen-

mates and, given the similarity in PFGE patterns of isolates from pen 19 and pen 20, may have occasionally transferred between pens. We have previously observed that a rifampin-resistant isolate introduced (via oral inoculation) into a subset of cattle in a feedlot pen was acquired and shed in the feces of all animals in the pen within 48 h of introduction (58). Transmission of this isolate between pens of cattle was observed only during the week immediately after the animals' entry to the feedlot, a period in which stress may have altered the stability of microbial populations within the digestive tract (3, 9). Coincidently, the increase in shedding on day 113 occurred during the beginning of the transition phase to the grain-based diet, a period of ecological shift in the makeup of the microbial populations within the digestive tract. Dissemination of *E. coli* O157:H7 (54) and antimicrobial-resistant *Campylobacter* strains among pens of feedlot cattle (29) has also been reported. Characterization of the selective pressures that give rise to widespread transmission of resistant clones within pens of livestock may provide insight into potential management strategies that may reduce the excretion of antibiotic-resistant bacteria into the environment. In addition, the temporal fluctuation of specific resistant clones within select animals may have implications for source tracking using genotypic or phenotypic antimicrobial-resistant traits. Consideration of such temporal variability should be taken into consideration when conducting studies in this area of research.

The higher prevalence of tetracycline-resistant *E. coli* among steers fed a grain-based diet compared to a silage-based diet was probably a result of increases in both the total number of *E. coli* isolated (Table 1) and the percentage of tetracycline-resistant *E. coli* within the total *E. coli* population (Table 2). Interestingly, the increase in total *E. coli* isolated in association with the grain-based versus the silage-based diet (~0.25 log) was observed in the steers fed antibiotics but not in the control group (no antibiotics). Others have reported that the transition of cattle from a forage-based diet to a grain-based diet increased the populations of *E. coli* by as much as 1 (55) to 3 (17) logs. With the exception of the TET-SUL treatment, the prevalence of animals shedding resistant *E. coli* increased when animals were fed the grain-based diet. Thus, diet is also an important factor to consider in analyzing antimicrobial-resistant bacteria from cattle, especially when phenotypes are compared, given that it may also skew data used for source tracking.

The dynamics within the intestinal environment that gave rise to the increased prevalence of tetracycline-resistant *E. coli* when steers were fed the grain-based diet as opposed to the silage-based diet are not easily defined. Dietary factors have been implicated previously in the development of antimicrobial-resistant *E. coli* populations in ruminants. A nonmedicated dietary supplement fed to dairy calves increased the prevalence of *E. coli* resistant to streptomycin, sulfadiazine, and tetracycline (31). In addition, indirect effects of the environment, such as cold stress, have been related to the increased prevalence of tetracycline- and ampicillin-resistant *E. coli* strains in swine, an effect that may have been attributable to changes in the level of feed consumption (39). Including high levels of grain in the diets for cattle reduces colonic pH and increases acid tolerance in *E. coli* populations (50). Exposure of *E. coli* to acidic conditions induces the production of a number of acid shock proteins, including membrane-bound transporters (8). Tetracycline antiporters coupled with proton influx is a common mechanism of tetracycline resistance in *E. coli* (47). The extent to which this mechanism of conferring resistance increases with declining pH (63) is not known, but it may have contributed to the increase in tetracycline resistance observed in the *E. coli* population when grain-based diets were fed. However, a myriad of other environmental stressors (e.g., bacteriocins, substrate availability, plant antimicrobials, osmolarity, etc.) within the intestinal tract may be altered as a result of a change from a silage-based to a grain-based diet. Defining the degree of linkage between the genes responsible for antibiotic resistance and those induced by various environmental stressors may enable a more accurate prediction of how changes in diet composition may impact the prevalence of antibiotic resistance in enteric bacteria.

Removing antibiotics from the diets of the feedlot cattle for 56 days during feeding of the silage-based diet and for 40 days during feeding of the grain-based diet did not significantly alter the prevalence of cattle shedding tetracycline- or ampicillin-resistant bacteria. A slight decline was noted in the prevalence of silage-fed cattle shedding tetracycline-resistant *E. coli*, when the TET-SUL agent was removed from the diet, but this decline was reversed completely, and the prevalence continued to increase during the feeding of the grain-based diet (Fig. 2). However, the increase in tetracycline resistance when the grain-based diet was fed was probably attributable to the diet itself, given that resistance levels also increased in the control animals. There are documented cases in which at least a portion of antibiotic-resistant bacterial populations reverted to an antibiotic-susceptible state (34) or their prevalence declined once selective antimicrobial pressures are removed, although in some instances this decline may require years (33, 45). In contrast, there have also been instances in which the withdrawal of antimicrobial drugs did not affect resistant populations (1, 32). A high prevalence of tetracycline-resistant *E. coli* in the digestive tract subsequent to the exclusion of tetracycline from the diet has been observed by other researchers, for a period of 126 months in swine (32) and 10 weeks in cattle (1). Reducing the use of antimicrobial agents may not decrease the prevalence of resistant microorganisms within a bacterial population if the presence of genetic elements conferring resistance is not a disadvantage for the resistant members (21). The maintenance of resistance may also be confounded by environmental factors. Genetic linkage between genes conferring resistance to metals (56) or other antibiotics (2) may maintain antimicrobial-resistant genes even in the absence of selective pressure. In the present study, removal of chlortetracycline from the diet was not a viable strategy for reducing the level of tetracycline-resistant *E. coli*.

In conclusion, upon entry into feedlots, cattle that had not previously been administered antimicrobial agents were shown to carry *E. coli* resistant to tetracycline and ampicillin. The administration of chlortetracycline in combination with sulfamethazine increased the prevalence of animals shedding tetracycline- and ampicillin-resistant *E. coli* and the numbers of resistant *E. coli* organisms shed. Feeding a grain-based diet to cattle increased both prevalence and numbers of tetracycline-resistant *E. coli*. For ampicillin-resistant *E. coli*, clonal dissemination seemed to occur readily between animals within a pen.

In total, the data reported here further highlight the complexity of detailing antimicrobial-resistant *E. coli* from feedlot cattle. Factors other than antimicrobial administration need to be considered when resistant bacteria are analyzed. Given the importance of antimicrobial resistance to human and veterinary medicine, linkages between environmental stressors and resistant genes should be investigated.

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