

## Diversity and Distribution of Commensal Fecal *Escherichia coli* Bacteria in Beef Cattle Administered Selected Subtherapeutic Antimicrobials in a Feedlot Setting<sup>∇†</sup>

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*Escherichia coli* strains isolated from fecal samples were screened to examine changes in phenotypic and genotypic characteristics including antimicrobial susceptibility, clonal type, and carriage of resistance determinants. The goal of this 197-day study was to investigate the influence of administration of chlortetracycline alone (T) or in combination with sulfamethazine (TS) on the development of resistance, dissemination of defined strain types, and prevalence of resistance determinants in feedlot cattle. Inherent tetracycline resistance was detected in cattle with no prior antimicrobial exposure. Antimicrobial administration was not found to be essential for the maintenance of inherently ampicillin-resistant and tetracycline-resistant (Tet<sup>r</sup>) *E. coli* in control animals; however, higher Tet<sup>r</sup> *E. coli* shedding was observed in animals subjected to the two treatments. At day 0, high tetracycline (26.7%), lower sulfamethoxazole-tetracycline (19.2%), and several other resistances were detected, which by the finishing phase (day 197) were restricted to ampicillin-tetracycline (47.5%), tetracycline (31.7%), and ampicillin-tetracycline-sulfamethoxazole (20.8%) from both treated and untreated cattle. Among the determinants, *bla*<sub>TEM1</sub>, *tet*(A), and *sul2* were prevalent at days 0 and 197. Further, *E. coli* from day 0 showed diverse antibiogram profiles and strain types, which by the finishing phase were limited to up to three, irrespective of the treatment. Some genetically identical strains expressed different phenotypes and harbored diverse determinants, indicating that mobile genetic elements contribute to resistance dissemination. This was supported by an increased linked inheritance of ampicillin and tetracycline resistance genes and prevalence of specific strains at day 197. Animals in the cohort shed increasingly similar genotypes by the finishing phase due to animal-to-animal strain transmission. Thus, characterizing inherent resistance and propagation of cohort-specific strains is crucial for determining antimicrobial resistance in cattle.

In recent years, the development of antimicrobial resistance (AR) in intensive livestock production has been increasingly reported (17). Consequently, the role of antimicrobial administration and the extent to which it affects the development of resistance in animals are receiving much attention. Antimicrobial usage for livestock can be for therapeutic, prophylactic, metaphylactic, or growth promotion purposes (26). Reportedly, 90% of the antimicrobials used in animal agriculture are for growth promotion and prophylaxis (26) and this widespread use is suggested to be an important contributor to the emergence, selection, and dissemination of antimicrobial-resistant bacteria, as indicated by several recent studies (9, 13, 28, 38). Antimicrobial-resistant bacteria, including *Escherichia coli*, are frequently isolated from the commensal gut flora of food animals (1, 2), and although the resistance they carry may not be a problem per se, the transfer of resistance elements to zoonotic pathogens inhabiting the gut has serious implications for animal and human health. Subtherapeutic use of antimicrobials is known to select for resistance (9, 14), but evidence suggests that development of resistance is far more complex

and that prevalence of resistant bacterial populations cannot be simply related to antimicrobial usage (29, 30) since antimicrobial withdrawal does not always reduce this resistance (5, 16). Further, the relationship between subtherapeutic usage and maintenance of resistance within the gut bacterial population has not been well characterized.

Commensal *E. coli* is often used as an indicator organism to assess the extent and type of resistance in the gastrointestinal tract since it plays a dynamic role in the ecology of multidrug-resistant bacteria and has been shown to be a reservoir of resistance (8, 42). Studies have demonstrated that young cattle have a higher prevalence of resistant fecal *E. coli* than older stock held on the same farm (12, 27), while carriage of ampicillin-resistant (Amp<sup>r</sup>) *E. coli* declines with calf age (22). Elucidating how young animals are affected by continuous subtherapeutic antimicrobial administration and defining the dynamics of acquisition of resistance in *E. coli* are essential for establishing the mechanism(s) of resistance transmission in feedlot cattle. Serotyping and resistance profiling have provided useful information relating to *E. coli* populations in cattle (20); however, these techniques have their limitations in establishing the movement of strain types. We used a pulsed-field gel electrophoresis (PFGE) fingerprinting method to discriminate between *E. coli* strains to understand the persistence of strain types and linked the derived genotypes to antibiogram profiles (ABGs; susceptibility testing) for assessing the move-

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ment of transferable resistance elements between strains. We chose to monitor Amp<sup>r</sup> and tetracycline (TE)-resistant (Tet<sup>r</sup>) commensal *E. coli* due to increasing resistance to these antimicrobials in humans.

Using healthy beef cattle maintained for 197 days in a feedlot setting, we investigated the influence of administration of dietary chlortetracycline alone (T) or in combination with sulfamethazine (TS) on resistance selection and distribution of resistance determinants. Further, we examined if resistance was disseminated by specific *E. coli* strain types between animals in the cohort. The concentrations of the antimicrobial agents used were similar to those of growth promoters used in the cattle industry. The specific objectives were addressed by monitoring (i) the shedding of Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* over time and assessing the effects of time and individual antimicrobial treatments on the two *E. coli* populations, (ii) the prevalence and stability of *E. coli* genetic and phenotypic diversity over time between treatments, and (iii) the distribution and emergence of common TE (*tet*), ampicillin (*amp*), and sulfonamide (*sul*) determinants in untreated versus treated beef cattle.

#### MATERIALS AND METHODS

**Animals, diets, and treatments.** The 240 crossbred steer calves (6 to 8 months old, weighing  $150 \pm 20$  kg) used in this study, which had no previous history of antimicrobial administration, were from Deseret Ranches (Raymond, Alberta, Canada). Animals were transported over 2 consecutive days by truck and upon arrival were housed in 24 pens at the Lethbridge Research Center experimental feedlot. Calves were arbitrarily assigned to one of the following treatments: (i) no antimicrobial agent ( $n = 50$ ; control), (ii) 350 mg head<sup>-1</sup> day<sup>-1</sup> chlortetracycline (Aureomycin-100G; Alphaforma Inc., Bridgewater, NJ) ( $n = 40$ ; T), (iii) 350 mg head<sup>-1</sup> day<sup>-1</sup> each chlortetracycline and sulfamethazine (Aureo S-700G; Alphaforma Inc.) ( $n = 50$ ; TS). Due to pen limitations, the T treatment could be replicated only four times and thus for uniformity, samples from 40 animals each from the control and treatment groups were processed. The antimicrobial agents were selected based on the commonality of their use in the Canadian feedlot industry and were fed at the concentrations recommended by the manufacturers. The treatments were arranged in a semirandomized complete block design, with each block consisting of a separate pen containing 10 steers. All antimicrobials supplemented the diet as top dressing which was mixed and spread manually over the feed surface during morning feeding, and animals were fed once daily. The pen setting was similar to any large industrial feedlot such that all animals could feed at the same time in their respective feed bunks. Water troughs were shared by animals in adjacent pens, and the treatments were arranged such that only animals that received the same treatment could drink from the same water troughs, preventing cross contamination.

For first 80 days, the steers were fed a typical forage-based background diet (70% barley silage, 25% barley grain, and 5% dry matter supplemented with vitamins and minerals) (Fig. 1). Thereafter, animals were shifted over a 21-day transition period from the background diet to a grain-based finishing diet (84% barley grain, 10% barley silage, and 5% supplements). The finishing diet was administered for an additional 124 days. The antimicrobials were administered continually for 197 days starting on day 0 (after fecal collection) and were withdrawn 28 days prior to slaughter. All animals were cared for according to the guidelines set out by the Canadian Council on Animal Care (7).

**Fecal sampling.** Fecal samples were collected from each animal nine times in time periods A, B, C (background diet), D (diet transition period), E, F, G, H (finishing diet), and I (off antimicrobials). For fecal sampling, cattle were restrained in a constraint device, and a deep fecal sample was obtained from the rectum via a fecal grab. All fecal samples were collected in sterile tubes, placed on ice, and transported within 1 h to the laboratory for plating. Fresh gloves were used for fecal collection from each animal.

**Isolation, identification, and enumeration of *E. coli*.** Each fecal sample (10 g wet weight) was aseptically placed in a sterile stomacher bag (Fisher Scientific, Ottawa, Ontario, Canada) containing 90 ml of 1× phosphate-buffered saline and mixed for 2 min (230 rpm, room temperature) with a Stomacher (Seward Ltd., Worthing, West Sussex, United Kingdom). The resulting slurries were 10-fold serially diluted, and 100 µl of the appropriate dilution was plated onto the following standard media for *E. coli* isolation in duplicate: MacConkey agar

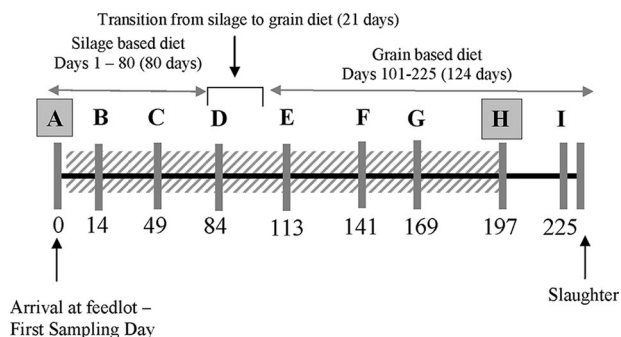


FIG. 1. Schematic representation of the time line of the experiment, outlining the arrival of animals, diets, and sampling days. The letters A to I represent sampling times (gray bars). The subtherapeutic antimicrobials were administered via top dressing (diagonal hatching) continuously for 197 days. Isolates from periods A (background diet) and H (finishing diet) were analyzed via susceptibility testing and PFGE and for resistance determinants. The numbers below the time periods represent the numbers of days into the trial. The drawing is not to scale.

containing no antibiotics (Difco, Sparks, MD) for total *E. coli* isolation, MacConkey agar containing ampicillin ( $32 \mu\text{g ml}^{-1}$ ) for Amp<sup>r</sup> *E. coli*, and MacConkey agar containing TE hydrochloride ( $16 \mu\text{g ml}^{-1}$ ) for isolation of Tet<sup>r</sup> *E. coli*. These antimicrobial concentrations are recommended breakpoints for *E. coli* according to the CLSI (11). The plates were incubated at 39°C for 24 h, and individual colonies were enumerated to calculate CFU counts by taking into account the number of observed colonies and the dilution factor. The numbers of CFU per gram (wet weight) of total, Amp<sup>r</sup>, and Tet<sup>r</sup> *E. coli* populations were calculated. These counts were used for statistical analysis in determining the effects of antimicrobial treatments and time on total, Amp<sup>r</sup>, and Tet<sup>r</sup> *E. coli* strain shedding, as well as to assess treatment-time interactions. Two isolates per animal from nonselective (MacConkey agar containing no antibiotics) plates were arbitrarily selected and confirmed to be *E. coli* with API 20E (bioMérieux Inc., Durham, NC). These positive isolates were streaked onto Trypticase soy agar (Difco) and grown at 39°C for 24 h. Each isolate was stored in 20% glycerol at -80°C until processed.

During standardization, we found that clonal types from individual animals were generally stable and hence one isolate per animal was used for characterization, which included PFGE analysis, susceptibility profiling, and characterization of resistance determinants. Thus, 80 isolates from the growing (period A,  $n = 40$ ) and finishing (period H,  $n = 40$ ) periods were analyzed for each group (control, T, and TS).

**Susceptibility testing.** The antimicrobial susceptibilities (phenotypes) of *E. coli* isolates from periods A and H to a panel of 12 antimicrobials was determined with a disk diffusion assay following CLSI standards (11). For this purpose, Mueller-Hinton II agar (Difco) was used and cells were harvested from the surface of the medium with a cotton swab after 24 h growth at 37°C. Cells were suspended in sterile saline (0.85% NaCl), cell density was adjusted to a 0.5 McFarland turbidity standard, and the diluted cells were plated. Following incubation, zone sizes were measured to two decimal points and used for quantitative analysis. Isolates resistant to two or more antimicrobials were defined as dual resistant or multiresistant, respectively. *E. coli* ATCC 25922 (American Type Culture Collection, Manassas, VA) was included in each assay as a control strain. Antimicrobial agents were tested with BD BBL Sensi-Disc antimicrobial susceptibility test discs (Becton Dickinson & Co., Sparks, MD) with the breakpoints (micrograms per milliliter) indicated as follows: amoxicillin-clavulanic acid (20 and 10, respectively), ampicillin (AMP; 10), amikacin (30), ceftriaxone (30), cefoxitin (30), ciprofloxacin (5), gentamicin (10), kanamycin (30), nalidixic acid (30), streptomycin (STR; 10), tetracycline (TE; 30), and trimethoprim-sulfamethoxazole (SXT; 1.25 and 23.75, respectively). The ABG of each isolate was established based on the resistance(s) observed.

**Genotyping.** PFGE was conducted to assess the genetic diversity of *E. coli* and to analyze strain transmission in the control and treatment groups during periods A and H. Isolates were subtyped on the basis of PFGE patterns (PPs) of XbaI-cleaved chromosomal DNA in accordance with the standard protocol established by the Centers for Disease Control and Prevention (PulseNet; Centers for Disease Control and Prevention, Atlanta, GA). DNA fragments were re-

TABLE 1. PCR primers and control strains used for identification of Amp<sup>r</sup>, Tet<sup>r</sup>, and Sul<sup>r</sup> determinants

Plasmid	Resistance gene	PCR primer sequence (5' → 3')	Amplicon size (bp)	Accession no.	Annealing temp (°C)	Reference
SU01	<i>pse1</i>	CGC TTC CCG TTA ACA AGT AC CTG GTT CAT TTC AGA TAG CG	419	M69058	60	18
SU05	<i>oxa1</i>	AGC AGC GCC AGT GCA TCA ATT CGA CCC CAA GTT TCC	708	AJ009819	60	18
SU07	<i>tem1</i>	TTG GGT GCA CGA GTG GGT TAA TTG TTG CCG GGA AGC	503	AF126482.1	60	18
pSL18	<i>tet(A)</i>	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	210	X61367	59.5	31
pRT11	<i>tet(B)</i>	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG	659	J01830	59.5	31
pBR322	<i>tet(C)</i>	CTT GAG AGC CTT CAA CCC AG ATG GTC GTC ATC TAC CTG CC	418	J01749	59.5	31
pSL106	<i>tet(D)</i>	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	787	L06798	59.5	31
pSL1504	<i>tet(E)</i>	AAA CCA CAT CCT CCA TAC GC AAA TAG GCC ACA ACC GTC AG	278	L06940	59.5	31
pJA8122	<i>tet(G)</i>	GCT CGG TGG TAT CTC TGC TC AGC AAC AGA ATC GGG AAC AC	468	S52437	59.5	31
PAT102	<i>tet(K)</i>	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT	169	S67449	59.5	31
pVB.A15	<i>tet(L)</i>	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267	U17153	59.5	31
pJ13	<i>tet(M)</i>	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406	X90939	59.5	31
pUOA1	<i>tet(O)</i>	AAC TTA GGC ATT CTG GCT CAC TCC CAC TGT TCC ATA TCG TCA	515	Y07780	59.5	31
pAT451	<i>tet(S)</i>	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667	C92946	59.5	31
pJIR39	<i>tetA(P)</i>	CTT GGA TTG CGG AAG AAG AG ATA TGC CCA TTT AAC CAC GC	676	L20800	59.5	31
pNFD13-2	<i>tet(Q)</i>	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	904	X58717	59.5	31
pBS5	<i>tet(X)</i>	CAA TAA TTG GTG GTG GAC CC TTC TTA CCT TGG ACA TCC CG	468	M37699	59.5	31
amr130	<i>sul1</i>	CGG CGT GGG CTA CCT GAA CG GCC GAT CGC GTG AAG TTC CG	433	AF071413	69	25
amr130	<i>sul2</i>	CGG CTC AAG GCA GAT GGC ATT GCG TTT GAT ACC GCG ACC CGT	285	M36657	69	25
rlo44	<i>sul3</i>	GAG CAA GAT TTT TGG AAT CG CTA ACC TAG GGC TTT GGA TAT	790	AJ459418	53	19

solved by electrophoresis on 1% SeaKem Gold agarose gels (Cambrex Bio-Science, Rockland, ME) with a contour-clamped homogeneous electric field DRII PFGE apparatus (Bio-Rad Laboratories, Mississauga, Ontario, Canada), with 0.5× Tris-borate-EDTA (BioBasics Inc., Edmonton, Alberta, Canada) as the running buffer, for 17 h at 14°C with a linearly ramped switching time from 2.2 to 55 s and a voltage of 6.0 V cm<sup>-1</sup>. Gels were stained with 0.5 mg liter<sup>-1</sup> ethidium bromide (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), destained in distilled water for 2 h, and photographed with an AlphaImager gel doc (version 4.1.0; Alpha Innotech FC, San Leandro, CA). XbaI-digested *Salmonella enterica* serovar Branderup H9812 was used as a marker and included in the first, middle, and last lanes of each gel to account for run-to-run variability. Comparison of digested profiles to identify restriction endonuclease digestion pattern clusters (REPCs) was performed with BioNumerics software, version 4.0 (Applied Maths, Austin, TX). Fingerprints were clustered by using the Dice coefficient evaluated by the unweighted-pair group method. A tolerance and optimization of 1% was allowed to account for gel differences. Each isolate was assigned a PP identification number. Isolates that were >90% related were considered highly related and were grouped as a cluster group. Patterns that did not fall into any particular REPC were also assigned a pattern identification number, and if they were detected only once during the trial they were considered unique.

**Characterization of resistance determinants.** Based on the ABGs *E. coli* isolates from periods A and H were characterized for genes responsible for ampicillin resistance (Amp<sup>r</sup>), TE resistance (Tet<sup>r</sup>), dual resistance (Amp<sup>r</sup> Tet<sup>r</sup>), and sulfonamide resistance (Sul<sup>r</sup>). Individual isolates that showed resistance

were screened for related resistance determinants (*amp*, *tet*, or *sul*). This was done by using multiplex PCR for three β-lactamase-encoding genes (*bla*<sub>OXA1</sub>, *bla*<sub>PSE1</sub>, and *bla*<sub>TEM1</sub>), 14 TE resistance-encoding alleles [*tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*, *tet(E)*, *tet(G)*, *tet(K)*, *tet(L)*, *tet(M)*, *tet(O)*, *tet(S)*, *tetA(P)*, *tet(Q)*, and *tet(X)*], and 3 *sul* alleles (*sul1*, *sul2*, and *sul3*). Details of primers, annealing temperatures, and amplicon sizes are provided in Table 1. Plasmids with known, sequenced genes were used as positive controls (Table 1).

To obtain a PCR template, individual *E. coli* isolates grown on MacConkey agar were resuspended in 25 μl of UltraPure DNase- and RNase-free water (Invitrogen, Burlington, Ontario, Canada) and lysed by boiling for 2 min. The suspension was centrifuged (12,000 × g for 2 min), and 1 μl supernatant was used as the template. The PCR mixture (25 μl) contained 1× reaction buffer and 1 U Platinum *Taq* polymerase (Invitrogen). The primers and deoxynucleoside triphosphate concentrations used were as specified in the original protocols cited below. The amplification conditions used for *amp* were as described previously (18). For amplification of *tet* genes, the conditions outlined by Ng et al. (31) were used. The PCR cycling conditions used for *sul1* and *sul2* were as described previously (25), while the amplification conditions used for *sul3* were those described by Hammerum et al. (19).

To ensure the absence of PCR inhibitors in lysate, a positive control that amplified a 200-bp fragment of the 16S rRNA gene was included in each PCR run with the primer sets and conditions described previously (25). All PCRs were performed on a PTC 100 thermocycler (MJ Research Inc., Watertown, MA), and each run included a negative control (no template DNA) and an appropriate positive control (control plasmid for the *tet*, *amp*, or *sul* determinant). All PCR

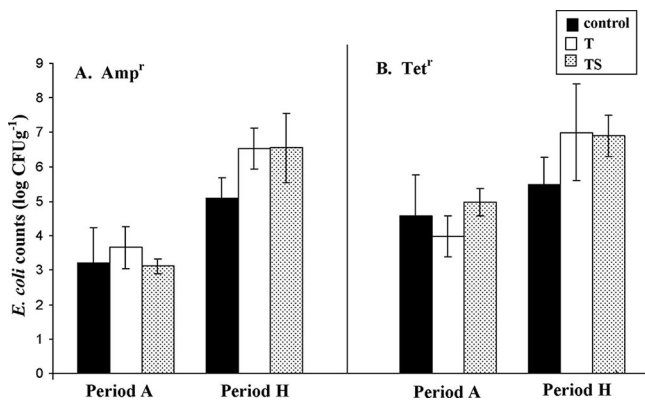


FIG. 2. Amp<sup>r</sup> (A) and Tet<sup>r</sup> (B) *E. coli* counts (log CFU g<sup>-1</sup> [wet weight]) in periods A and H with no antibiotic treatment (control), 350 mg head<sup>-1</sup> day<sup>-1</sup> chlortetracycline (T), and 350 mg head<sup>-1</sup> day<sup>-1</sup> each chlortetracycline and sulfamethazine (TS).

products (18  $\mu$ l) were resolved on 1.5% agarose gels containing ethidium bromide by standard procedures. A 100-bp DNA ladder (MBI Fermentas, Burlington, Ontario, Canada) was used to determine product sizes.

**Statistical analysis.** *E. coli* CFU counts were averaged from replicate plates. The counts were log transformed, and the mean and variance were calculated for each pen before performing the weighted analyses, with the inverse of the variance for each pen being used as the weight. For assessing treatment effect, counts from four time periods (A, D, E, and H) were analyzed for the total, Amp<sup>r</sup>, and Tet<sup>r</sup> *E. coli* populations. For assessing time effects, the numbers of CFU were analyzed by a weighted analysis of variance by the MIXED procedure (36) with treatment, time, and their interaction in the model as fixed effects and pen nested in treatment as the random effect. Time was treated as a repeated-measures effect to account for potential correlations among the various times. Various types of variance-covariance structures were fitted, and the one with the lowest Akaike information criterion value was used for the final analysis. Linear and quadratic orthogonal polynomials were used to check the time effect for trends. The UNIVARIATE procedure was used to check the residuals for normality and for potential outliers. When an outlier was detected, it was removed before the final analysis was performed. *P* values were used to estimate significant differences between various treatments over time, and *P* values of  $\leq 0.05$  were considered to indicate significant differences.

Cluster analysis was performed on PFGE clonal types by jackknife comparison between REPCs within a treatment. The resulting PPs were characterized by using the criteria described by Tenover et al. (40).

## RESULTS AND DISCUSSION

**Characterization of Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* strain.** Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* strains were detected throughout this study. Both Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* strain numbers were higher in period H than in period A (Fig. 2), in contrast to a previous study, in which a decline in Amp<sup>r</sup> *E. coli* counts with animal age was observed (22). Tet<sup>r</sup> *E. coli* counts were higher than Amp<sup>r</sup> *E. coli* counts for the control and treatment groups in periods A and H (Fig. 2). Overall, higher numbers of Tet<sup>r</sup> and Amp<sup>r</sup> *E. coli* bacteria were shed in period H when either T or TS was administered compared to the control. However, continued detection of Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* in time period H in control animals suggests that antimicrobial administration may not be the only factor contributing to continued inherent resistance past day 0 and other aspects such as a change in animal diet from forage to grain could help in sustaining resistance, as also suggested previously (41). A drop in pH in the rumen and the gastrointestinal tract occurs when cattle are shifted from a forage-based background diet to a grain-based finishing diet.

Increasing evidence corroborates that a low pH supports AR *E. coli* proliferation and implicates diet as playing a crucial role in controlling bacterial populations from animals with no prior antimicrobial exposure (4). Our observations are corroborated by previous studies in which Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* strains were frequently detected in poultry, swine, and cattle despite the restricted use of antimicrobials (18, 24).

As in any commercial feedlot, antimicrobial administration was terminated 28 days prior to animal slaughter. In order to assess the effect of antimicrobial withdrawal, comparison of counts in period H versus those in period I indicated a marginal ( $< 0.5$  log CFU g<sup>-1</sup>) decline in Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* strains in the T and TS groups ( $P > 0.5$ ) (data not shown). This indicates that Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli* can persist for at least 28 days in the gastrointestinal tract and could be disseminated during animal slaughter. These observations are in agreement with other studies in which withdrawal of antimicrobials did not necessarily result in an immediate decrease in the prevalence of resistant bacteria (10, 16, 32, 34).

Periods A, D, E, and H were used to assess the effects of antimicrobial treatment and time on *E. coli* counts. Least-square differences revealed significant treatment effects on Tet<sup>r</sup> *E. coli* ( $P < 0.001$ ), and a significant effect of time on all three (total, Amp<sup>r</sup>, and Tet<sup>r</sup>) *E. coli* populations ( $P < 0.001$ ) was observed. Analysis revealed a significant treatment-time interaction for Tet<sup>r</sup> ( $P < 0.001$ ) but not for Amp<sup>r</sup> *E. coli* ( $P = 0.9532$ ). Orthogonal contrasts revealed significant linear relationships with time for Tet<sup>r</sup> *E. coli* in the control ( $P < 0.001$ ) and TS ( $P < 0.001$ ) groups and a significant quadratic relationship for the T group ( $P < 0.001$ ). Although the highest Tet<sup>r</sup> *E. coli* increase per week from period A period to H was observed in the TS group (0.14 log CFU g<sup>-1</sup> week<sup>-1</sup>), followed by the control group (0.11 log CFU g<sup>-1</sup> week<sup>-1</sup>), the highest counts (absolute numbers) of Tet<sup>r</sup> *E. coli* bacteria shed (CFU per gram) were observed in the T group and followed the trend T > TS > control. We found limited information in the literature on the effect of TE administration on Tet<sup>r</sup> *E. coli* shedding over time.

**Susceptibility profiles.** All of the isolates examined in this study carried determinants of resistance to at least one of the antimicrobials tested, as also observed previously in domesticated animals (38, 39). The antibiogram diversity, irrespective of the treatments, decreased as the trial progressed and animals adapted to a finishing diet (period H) (Fig. 3C). Within the control population in period A, isolates displayed 11 ABGs, with SXT TE being a common phenotype found in 22.5% of the isolates (Table 2). By period H, only three ABGs were recorded (Fig. 3C), with AMP TE detected in 50% of the isolates (Table 2). Isolates from the T treatment group in period A also displayed 11 ABGs, which decreased to 3 by period H (Fig. 3C). For this treatment, however, higher frequencies of detection of TE and SXT TE (30 and 27.5% of the isolates) in period A and AMP TE and TE (37.5% of the isolates for both phenotypes) in period H were observed (Table 2). In the TS treatment group, period A isolates displayed 13 ABGs, which also decreased to 3 by period H (Fig. 3C); the most common phenotype in period A was TE (30% of the isolates), which by period H had changed to AMP TE (55% of the isolates) (Table 2). In the literature, we did not find any evidence of a decrease in phenotypic diversity with animal age;

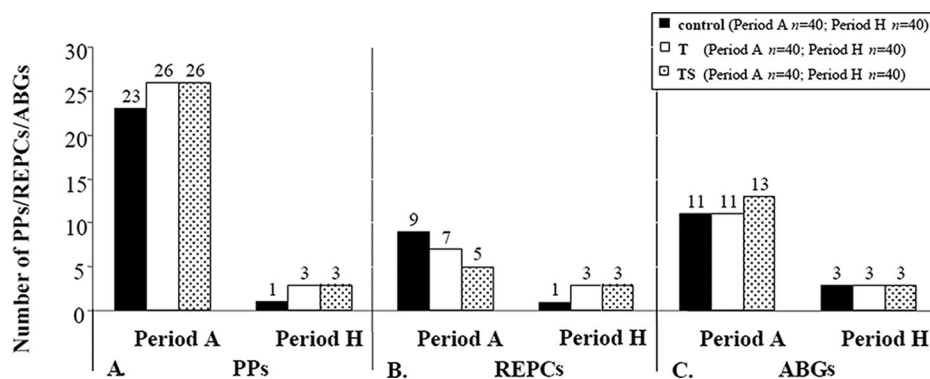


FIG. 3. Numbers of PPs (A) REPCs (B), and ABGs (C) in tested isolates from periods A and H within the control group and the T and TS treatment groups.

however, a higher prevalence of resistant fecal *E. coli* in younger than older animals held within the same farm has been previously reported (3, 20, 21, 27, 30). Khachatryan et al. (27) have suggested that the intestinal physiology of young animals favors certain niche-specific clones which are retained in older animals. Environmental sources have also been implicated as playing a role in the inoculation of particular strain types, with active competition in the bovine gut leading to the expansion of these niche-specific strains (28). We know for certain that the animals in this study were not exposed to any antimicrobials prior to arrival at our feedlot and that the 6- to 8-month-old animals had diverse phenotypes on day 0. Therefore, animal-to-animal transmission of selected phenotypes and strains (discussed later) seems to better fit predominate detection of TE and AMP TE by period H in the control and treatment groups. A fitness advantage of strains carrying these prevalent phenotypes, however, cannot be ruled out.

Previous studies of cattle indicate a higher prevalence of certain phenotypes, which varies from study to study but is found to be consistent within a trial. Certain core phenotypes were found to be common in this study, irrespective of the treatment, such as SXT TE (19.2%) and TE (26.7%) in period A and TE (31.7%) and AMP TE (47.5%) in period H (Table 2). These commonly detected phenotypes were different from those reported in previous investigations. For example, STR TE, STR SXT, and SXT TE were found in 60.6 to 77.3% of the isolates and STR SXT TE was found in 60.4% of the isolates from calves (23). However, STR SXT TE, another common phenotype recovered from cattle (28), was not detected in our study in any time period. We found a significant increase in the proportion of isolates with the phenotype AMP TE ( $P = 0.026$ ) over time but no significant difference in the other phenotypes examined, including TE ( $P = 0.315$ ). Data from period H showed significant acquisition of ampicillin resistance, which was always linked to either TE or SXT resistance, irrespective of the treatment (Table 2). We also observed sustained TE resistance in the control, T, and TS groups even though resistance to other antimicrobials was not detected by period H. Persistent detection of the TE resistance phenotype in control animals also supports our data on Tet<sup>r</sup> *E. coli* counts in periods A and H.

**Temporal distribution of strains.** In order to better understand strain prevalence, isolates were genotyped and corre-

lated to ABGs. Given a similarity coefficient of 90% (Dice index), PFGE data revealed 44 distinct PPs among the 240 isolates examined (Fig. 4). When comparing PFGE data in period A, some PPs (3 and 11) were detected in all of the treatment groups whereas others were specific to a particular treatment group. Further, some isolates within the control ( $n = 13$ ), T ( $n = 20$ ), and TS ( $n = 21$ ) treatment groups did not cluster into any REPC. Unique isolates ( $n = 8$ ) were also detected (Fig. 4).

Within the control group, 23 different PPs clustered into nine distinct REPCs in period A and, interestingly, by period H were limited to PP 30 and formed a single REPC (Fig. 3A and B and 4A). Close examination of PP 30 revealed that this genotype was shared by two distinct phenotypes: TE and AMP TE. Based on the strain and resistance gene analysis, we found that this particular strain had acquired the additional ampicillin resistance over time (as discussed later). For the T treatment group, 26 different PPs in period A clustered into seven REPCs, which by period H were reduced to 3 predominant PPs (30, 35, 41) and REPCs (Fig. 3A and B and 4B). Similarly, isolates from period A in the TS treatment group showed 26 different PPs, which clustered into five REPCs and by period H were limited to three PPs (30, 36, 41) and REPCs (Fig. 3A and B and 4C).

The reduction in the numbers of PPs and REPCs correlates well with the limited number of ABGs by period H, indicating overall lower strain diversity by the finishing phase in cattle in a feedlot setting. This decrease in genetic diversity was not a treatment effect since it was also observed in animals in the control group. Instead, it appears to be due to strain selection and propagation of selected strain types via animal-to-animal transmission. In order to validate this strain transmission hypothesis, the PFGE types of individual animals were tracked over time and *E. coli* isolates from animals in the same pens were observed to cluster into related REPCs, indicative of sufficient movement of *E. coli* between animals. We found that animals within a pen, among different pens, and also within this cohort started shedding *E. coli* strains with similar genotypic characteristics over time. Given these results, environmental sources are speculated to play a role in strain transmission among cattle. The animals in the control and treatment groups were housed spatially separate from each other, and no contact was possible between animals on different treatments and even

TABLE 2. Frequencies of detection of particular antibiogram patterns during periods A<sup>a</sup> and H<sup>a</sup> in control group and T and TS treatment groups

Group (no. of animals) and ABG <sup>b</sup>	Frequency	% of isolates within:		ABG <sup>b</sup>	Frequency	% of isolates within:	
		Treatment group (n = 40)	Period A <sup>c</sup> (n = 120)			Treatment group (n = 40)	Period H <sup>c</sup> (n = 120)
Control (40)							
SXT TE	9	22.5	19.2	AMP TE	20	50	47.5
TE	8	20	26.7	TE	16	40	31.7
AMP SXT TE	6	15	7.5	AMP TE SXT	4	10	20.8
AMC AMP CIP TE	5	12.5	7.5				
AMP	4	10	5				
FOX SXT TE	3	7.5	5				
AMC CIP TE	1	2.5	1.7				
AMP CIP SXT	1	2.5	3.4				
AMP CF FOX	1	2.5	2.5				
AMP SXT	1	2.5	1.7				
AMP TE	1	2.5	3.4				
T (40)							
TE	12	30		AMP TE	15	37.5	
SXT TE	11	27.5		TE	15	37.5	
AMC TE	3	7.5	3.4	AMP TE SXT	10	25	
AMP CIP SXT	3	7.5					
FOX SXT TE	3	7.5					
CIP TE	3	7.5	8.3				
AMC CIP TE	1	2.5					
AMP CF	1	2.5	1.7				
AMP CF FOX	1	2.5					
AMP TE	1	2.5					
CF CIP TE	1	2.5	0.8				
TS (40)							
TE	12	30		AMP TE	22	55	
CIP TE	7	17.5		AMP TE SXT	11	27.5	
AMC AMP CIP TE	4	10		TE	7	17.5	
AMP SXT TE	3	7.5					
SXT TE	3	7.5					
AMC AMP	2	5	1.7				
AMP	2	5					
AMP TE	2	5					
AMP CF FOX	1	2.5					
AMC TE	1	2.5					
AMP CF	1	2.5					
AMP CF TE	1	2.5	0.8				
AMP SXT	1	2.5					

<sup>a</sup> n = 120.<sup>b</sup> Abbreviations: ABG, antibiogram profile; AMC, amoxicillin-clavulanic acid; AMP, ampicillin; AMK, amikacin; CF, ceftriaxone; FOX, cefoxitin; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole.<sup>c</sup> Takes into account all three treatments.

among some replicates of the same treatment. Some nose-to-nose and body contact between animals in adjacent pens and within the same pen could be responsible for possible strain transmissions, but this limited contact cannot explain the presence of the same strain type in spatially separate pens. The animal-to-animal transmission is further validated by the diverse PPs of animals in period A which had little to no chance of strain transmission at day 0. It has also been established by other studies that the administration of antimicrobials selects for resistant bacteria which are subsequently transferred through contaminated food or water and that the feedlot environment is a crucial source of new strains (23). Similarly, in our study we found that antimicrobial administration to beef cattle selects for resistant *E. coli* and we speculate the feedlot environment plays a critical role in resistance dissemination.

The exact route of strain transmission was not characterized in this study, but fecal material is considered a possible source. Our results are in contrast to those of a previous study in which animals in interspersed pens maintained distinct *E. coli* populations (28).

We recognize that using a single isolate per animal may have its limitations. Therefore, upon observing similar clonal types in animals on different treatments, we PFGE typed an additional isolate from each animal in the T and TS groups in period H. We found that these supplementary isolates grouped in the already described phenotypes and PPs. In the past, single isolates have been used to assess the temporal diversity of antimicrobial-resistant *E. coli* without compromising the trends over time (6, 23). In addition, the repeated measure offered by four replicates in each treatment group gave us a

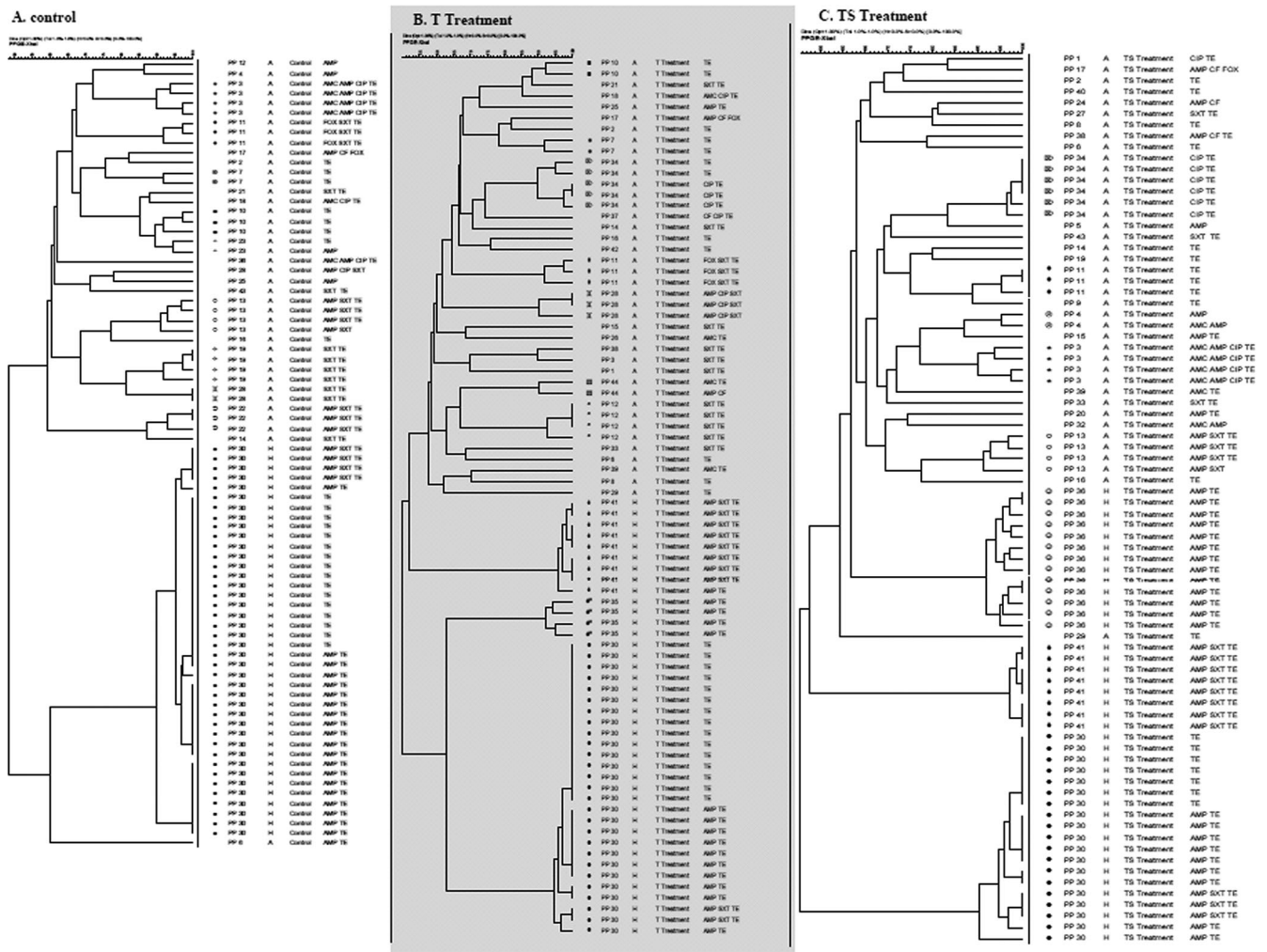


FIG. 4. Dendrograms showing the relationship between isolates shed in period A (day 0;  $n = 40$ ) and those shed in period H (day 197;  $n = 40$ ) by the control (A), T treatment (B), and TS treatment (C) groups. PPs, time periods, ABGs, and REPCs (>90% banding similarity) in the indicated time periods are shown. REPCs are separated by different symbols among the three treatment groups.

statistically significant sample size, confirming the trends, and showed that an increase in sample size did not result in an increased genotype number, as also previously observed (23).

**Distribution of resistance genes in *E. coli*.** Isolates from periods A and H displaying ampicillin resistance ( $Amp^r$ ,  $n = 19$ ), TE resistance ( $Tet^r$ ,  $n = 116$ ), dual resistance ( $Amp^r Tet^r$ ;  $n = 105$ ), or SXT resistance ( $Sul^r$ ,  $n = 69$ ) upon susceptibility testing were characterized for resistance alleles in order to understand the distribution of genes and their acquisition over time. In many cases, we found that genotypically similar strains did not necessarily carry the same resistance determinants.

**$\beta$ -Lactamase genes.**  $Amp^r$  alone was observed exclusively in 19 of the period A isolates and was never observed among isolates from period H (Table 3). Upon testing for  $\beta$ -lactamase genes, we found that  $bla_{TEM1}$  was present in 14 of these isolates while  $bla_{PSE1}$  was detected in the remaining 5 (Table 3).  $bla_{TEM1}$  has been previously detected as a predominant allele in beef cattle (18).

**TE resistance-encoding genes.** Alleles  $tet(K)$ ,  $tet(L)$ ,  $tet(M)$ ,  $tet(O)$ ,  $tet(P)$ ,  $tet(Q)$ ,  $tet(S)$ ,  $tet(X)$ , and  $tetA(P)$  were never

detected, and some of these have only been found in gram-positive bacteria.  $tet(A)$  was the predominant allele detected in period A (44.9%) and period H (36.8%);  $tet(B)$  was also prevalent but at lower frequencies in periods A (19.2%) and H (18.4%) (Table 3). Taking into consideration both periods A and H, the  $tet(A)$  and  $tet(B)$  resistance alleles accounted for 42.2 and 31.9% of the TE resistance among isolates. Overall, the frequency of detection of the  $tet$  alleles was  $tet(A) > tet(B) > tet(C)$ , being detected in 40.7, 17.6, and 11.3% of the total isolates, respectively. Similar to our results, Guerra et al. (18) found  $tet(A)$  and  $tet(B)$  to be the predominant alleles detected in 66 and 42% of the isolates examined from cattle, swine, and poultry. Like the ABGs, the prevalence of individual determinants responsible for specific resistances varies from study to study. For instance,  $tet(B)$  was the predominant determinant detected in 93% of the isolates and  $tet(A)$  was detected to a lesser extent (7%) in dairy cattle (37).

Several  $Tet^r$  isolates ( $n = 32$ ) from periods A and H contained more than one  $tet$  allele (dual  $tet$  resistance) (Table 3).  $tet(A) tet(B)$  was detected in periods A (11.5%) and H

TABLE 3. PCR detection of Amp<sup>r</sup> and Tet<sup>r</sup> determinants in isolates from periods A and H<sup>a</sup>

Phenotype tested and allele(s)	No. of isolates					
	Amp <sup>r</sup> (n = 19)		Tet <sup>r</sup> (n = 116)		Amp <sup>r</sup> Tet <sup>r</sup> (n = 105)	
	Period A (n = 19)	Period H (n = 0)	Period A (n = 78)	Period H (n = 38)	Period A (n = 23)	Period H (n = 82)
Amp <sup>r</sup>						
<i>bla</i> <sub>PSE1</sub>	5	0	NA <sup>d</sup>	NA	4	27
<i>bla</i> <sub>OXA1</sub>	0	0	NA	NA	0	0
<i>bla</i> <sub>TEM1</sub>	14	0	NA	NA	19	55
Tet <sup>r</sup>						
<i>tet</i> (A) <sup>b</sup>	NA	NA	35	14	7	34
<i>tet</i> (B) <sup>b</sup>	NA	NA	15	7	4	13
<i>tet</i> (C) <sup>b</sup>	NA	NA	8	5	3	9
<i>tet</i> (A) <i>tet</i> (B) <sup>c</sup>	NA	NA	9	5	4	11
<i>tet</i> (B) <i>tet</i> (C) <sup>c</sup>	NA	NA	5	5	2	4
<i>tet</i> (A) <i>tet</i> (C) <sup>c</sup>	NA	NA	6	2	3	11

<sup>a</sup> Isolates that displayed Amp<sup>r</sup>, Tet<sup>r</sup>, or Amp<sup>r</sup> Tet<sup>r</sup> dual resistance upon susceptibility testing were selected for resistance allele characterization.

<sup>b</sup> Single resistance.

<sup>c</sup> Dual resistance.

<sup>d</sup> NA, not applicable.

(13.2%); combined *tet*(B) *tet*(C) and *tet*(A) *tet*(C) were also observed.

An increase in the number of dual-resistant Amp<sup>r</sup> Tet<sup>r</sup> isolates from period A (21.9%) to period H (78.1%) and a marked reduction in the number of isolates carrying a single resistance, either Amp<sup>r</sup> or Tet<sup>r</sup> alone, in period H was clearly observed and not restricted to a few individual animals or pens. These results are corroborated by ABGs, which showed an increased prevalence of isolates carrying AMP TE. Of the 105 isolates which tested positive for combined Amp<sup>r</sup> Tet<sup>r</sup> (Table 3), 23 belonged to period A and 82 belonged to period H. *tet*(A) was detected frequently in periods A (30.4%) and H (41.5%), while *bla*<sub>TEM1</sub> conferred ampicillin resistance in periods A (82.6%) and H (67.1%). *bla*<sub>OXA1</sub> and *bla*<sub>PSE1</sub> were never detected within these dual-resistant isolates. These results indicate an increase in combined *tet*(A) and *bla*<sub>TEM1</sub> acquisition by period H. As noted via genotyping previously, predominant detection of PP 30 in period H with phenotype AMP TE or TE suggested that this strain type either acquired or lost Amp<sup>r</sup> over time to display this phenotype. Resistance gene distribution supports the acquisition of Amp<sup>r</sup> within PP 30 (Fig. 4; Table 3). Other investigators have reported a similar genetic linkage of antimicrobial drug resistance genes (39). As observed for Tet<sup>r</sup> single resistance, Amp<sup>r</sup> Tet<sup>r</sup> isolates also

showed an increase in dual TE resistance genes, viz., *tet*(A) *tet*(B), *tet*(B) *tet*(C), and *tet*(A) *tet*(C), in periods A to H (Table 3).

**Sulfonamide genes.** Of the isolates examined from periods A and H, 69 exhibited Sul<sup>r</sup> (Table 4). *sul2* was detected at a higher frequency (47.8%) than *sul1* (17.4%) (Table 4). Similar higher *sul2* than *sul1* detection has been reported earlier in humans and swine (19) and attributed to a fitness advantage in clinical human *E. coli* (15). *sul3* was never detected in this study but has been previously reported in *E. coli* from pigs (18, 19, 33, 35). Dual *sul1 sul2* genes were also observed in isolates from both periods A (31.9%) and H (40%) (Table 4).

In conclusion, results from the present study indicate that fecal *E. coli* populations in beef cattle in a feedlot setting are temporally variable and that antimicrobial selection pressure is not essential for the maintenance of inherent Amp<sup>r</sup> and Tet<sup>r</sup> *E. coli*. Nevertheless, usage of chlortetracycline alone or in combination with sulfamethazine results in higher Tet<sup>r</sup> *E. coli* shedding. Inherent resistance in other animals of the cohort affects resistance dissemination and strain acquisition. By the finishing phase, an increased combined ampicillin and TE resistance was observed and cattle in the cohort shed *E. coli* with resistance limited to the AMP SXT TE, AMP TE, and TE phenotypes, irrespective of treatment. Genotyping results confirmed strain movement from animal to animal in the cohort to be responsible for this limited diversity and supports the hypothesis that strain transmission among animals plays a crucial role in transmitting AR. The transmission of selected antimicrobial-resistant *E. coli* genotypes indicates that feedlot farms are important reservoirs of resistance. Detection of inherent and acquired resistance in *E. coli* from beef cattle in this study proves the importance of monitoring as an essential component of AR management strategies.

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TABLE 4. PCR detection of Sul<sup>r</sup> determinants in isolates from periods A and H that displayed Sul<sup>r</sup> upon susceptibility testing

Allele(s)	No. of resistant isolates (total n = 69)	
	Period A (n = 44)	Period H (n = 25)
<i>sul1</i> <sup>a</sup>	7	5
<i>sul2</i> <sup>a</sup>	23	10
<i>sul3</i> <sup>a</sup>	0	0
<i>sul1 sul2</i> <sup>b</sup>	14	10

<sup>a</sup> Single resistance.

<sup>b</sup> Dual resistance.



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