Longitudinal Characterization of Resistant *Escherichia coli* in Fecal Deposits from Cattle Fed Subtherapeutic Levels of Antimicrobials[▽]

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Model fecal deposits from cattle fed or not fed antimicrobial growth promoters were examined over 175 days in the field for growth and persistence of total *Escherichia coli* and numbers and proportions of ampicillin-resistant (Amp^r) and tetracycline-resistant (Tet^r) *E. coli*. In addition, genotypic diversity and the frequency of genetic determinants encoded by Amp^r and Tet^r *E. coli* were investigated. Cattle were fed diets containing chlortetracycline (44 ppm; A44 treatment group), chlortetracycline plus sulfamethazine (both at 44 ppm; AS700 treatment group), or no antibiotics (control). Fecal deposits were sampled 12 times over 175 days. Numbers of Tet^r *E. coli* in A44 and AS700 deposits were higher ($P < 0.001$) than those of controls and represented up to 35.6% and 20.2% of total *E. coli*, respectively. A time-by-treatment interaction ($P < 0.001$) was observed for the numbers of Tet^r and Amp^r *E. coli*. Except for Amp^r *E. coli* in control deposits, all *E. coli* numbers increased ($P < 0.001$) in deposits up to day 56. Even after 175 days, high Tet^r *E. coli* numbers were detected in A44 and AS700 deposits [5.9 log$_{10}$ CFU (g dry matter)$^{-1}$ and 5.4 log$_{10}$ CFU (g dry matter)$^{-1}$, respectively]. *E. coli* genotypes, as determined by pulsed-field gel electrophoresis, were diverse and were influenced by the antimicrobial growth promoter and the sampling time. Of the determinants screened, bla_{TEM1}, $tetA$, $tetB$, $tetC$, $sul1$, and $sul2$ were frequently detected. Occurrence of determinants was influenced by the feeding of antimicrobials. Fecal deposits remain a source of resistant *E. coli* even after a considerable period of environmental exposure.

In North America antibiotics are widely used in beef cattle production as prophylactics or antimicrobial growth promoters (AGP). Used in this manner, antibiotics are generally administered in the diet either at times of high disease risk or on a continuous basis to improve feed efficiency. Employment of AGP in this manner may increase the prevalence of commensal antimicrobial-resistant (AR) bacteria (1, 41).

There is evidence that resistant bacteria can be transferred from livestock to humans (5, 39, 55), and consequently the use of AGP has raised public health concerns. While the modes of transmission of AR bacteria and gene determinants are complex (33), survivability in agriculture-related matrices may be a critical factor in their dissemination (54). Most research on the persistence of AR bacteria in livestock waste has focused on large-scale management systems, such as land-applied manure (20, 44) or storage lagoons and pits (28, 49). In some instances, these systems have been shown to decrease the survival of select bacteria and the presence of AR genes (35, 51). Nevertheless, there is limited knowledge of the fate of AR bacteria or resistance determinants in feces shed from individuals, such as the fecal deposits that are formed in feedlot pens on intensively managed farms. Previous work has shown that fecal deposits provide an environment that is conducive to the growth and survival of pathogenic and commensal *Escherichia coli* (4, 48, 57). Because cattle fed AGP excrete antimicrobial residues in their feces (3), the residues may exert a selection pressure on bacteria after deposition of feces and potentially confer a growth advantage to resistant bacteria.

The objective of the current study was to investigate both the longitudinal phenotypic and genotypic ecologies of AR *E. coli* in fecal deposits arising from groups of cattle administered either no AGP or one of two tetracycline-based AGP that are commonly used in the North American industry. We focused on ampicillin-resistant (Amp^r) and tetracycline-resistant (Tet^r) *E. coli* because both AGP treatments contained tetracycline, and we have previously found a link between the use of tetracycline-based AGP and Amp^r *E. coli* (1). We hypothesized that resistant *E. coli* would be more numerous in fecal deposits from animals previously fed AGP.

MATERIALS AND METHODS

Animals and treatments. A complete history of antimicrobial administration to the steers generating the fecal deposits was known and controlled. Ninety crossbred steers (6 to 8 months old, weighing 150 ± 20 kg) were randomly assigned to nine pens at the Lethbridge Research Centre feedlot (Lethbridge, Alberta). 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. Three pens (10 steers per pen) were assigned to each of three treatments: (i) control, no antibiotics; (ii) chlortetracycline (44 ppm; fed as Aureomycin-100 G; Alpharma; treatment denoted A44); (iii) chlortetracycline and sulfamethazine (each at 44 ppm; fed as Aureo S-700 G; Alpharma, Inc., Bridgewater, NJ; treatment denoted AS700). These antibiotics and concentrations were selected on the basis of conventional practices in the Canadian beef industry. Adjacent pens in the feedlot were supplied with 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 administered antimicrobials for 197 days, starting on the day of arrival to the feedlot, up to the point of fecal collection for construction of deposits. At the time of fecal deposit setup, steers had been fed a concentrate-based diet for the previous 96 days that consisted of the following (dry-matter [DM] basis): 85% barley, 10% barley silage, and 5% supplement which contained

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calcium carbonate (34.8%), zinc sulfate (28.4%), manganous sulfate (14.6%), copper sulfate (10.3%), selenium (5.0%), vitamin E (4.7%), vitamin A (1.7%), vitamin D (0.2%), ethylenediamine dihydroiodide (0.1%), and cobalt sulfate (0.1%) (wt/wt basis). The steers were fed once daily in a manner that ensured that all feed was consumed daily. 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 feed delivery. Steers assigned to the control treatment had no access to medicated feed at any time during the experiment. All cattle were cared for according to the guidelines of the Canadian Council on Animal Care (12).

Fecal deposit preparation and sampling. For each pen, individual fecal samples ($n = 10$) were collected from each animal and mixed uniformly into a single composite (approximately 24 kg). The fecal material was collected in a manner that avoided feces that had contacted the ground and was added to the composite mixture within 1 min after defecation. Each composite mixture was then divided into duplicate artificial fecal deposits contained in metal pans (50 by 50 by 5 cm) to prevent possible contamination between treatments. The depth of the fecal deposits was ~5 cm. The bottoms of the pans were perforated to allow water to drain to the subsoil in the event of rainfall. In total, 18 fecal deposits (2 replicates per pen) were prepared. The deposits were randomly placed outside on 1 March in two adjacent rows with 50-cm spacing between deposits. Deposit volume was predicated on the need to provide periodic samples over 175 days.

Fecal deposits were sampled after 0, 7, 14, 28, 42, 56, 70, 84, 98, 112, 126, and 175 days of environmental exposure. Deposits were sampled using sterile 60-ml syringes modified by cutting the end of the barrel of the syringe. At each sampling, two subsamples (~15 g) 3 cm apart were collected and pooled. The two samples were collected by inserting the syringe into the bottom of the deposit and expelling the sample into a sterile cup using the plunger. Samples were immediately taken to the lab for processing. Average daily temperature and precipitation data for the Lethbridge Research Centre were obtained from the Agriculture and Agri-Food Canada weather observation station that was located 100 m from the site.

Microbial analysis. Fecal material from each deposit was thoroughly mixed, and a subsample (5 g) was weighed into a pan and placed in a 105°C oven for 24 h to determine DM. A second sample (1 g) was placed in a stomacher bag (10.5 by 15 cm) and mixed with 9 ml of 0.05 M sodium phosphate buffer (pH 7.0). Bags were stomached for 2 min (setting 8; Interscience Mini-Mix), and the contents were used in a 1:10 dilution series with sodium phosphate buffer. Aliquots (100 μl) from selected dilutions were plated in duplicate on MacConkey agar (Difco/Becton Dickinson, Sparks, MD) containing no antibiotics (MAC) and on MAC amended with ampicillin (32 μg ml^{-1}) (MAC+AMP) or tetracycline hydrochloride (16 μg ml^{-1}) (MAC+TET). Plates were incubated for 18 to 24 h at 37°C before lactose-positive colonies were counted. Concentrations of antibiotics used in the MAC+AMP and MAC+TET plates were set at the breakpoint standards for defining resistance as described by the Clinical and Laboratory Standards Institute (15), and isolates that grew on these plates were considered resistant. Colony counts on each medium were reported as $\log_{10}$ CFU (g DM)$^{-1}$. The percentage of *E. coli* isolates exhibiting resistance to the antibiotic concentrations in selective plates was calculated as follows: (number of colonies on the antibiotic-supplemented MAC plates/total colonies counted on nonselective MAC) × 100.

Four isolates from MAC and when possible up to six isolates from MAC+AMP and MAC+TET plates were selected, streaked onto Trypticase soy agar (Difco), and incubated at 37°C for 24 h. Colonies on the Trypticase soy agar plates were transferred and stored at −80°C in 20% (vol/vol) glycerol in brain heart infusion medium (Difco). AR isolates selected for pulsed-field gel electrophoresis (PFGE) (see below) were again tested for growth on plates containing the antimicrobial to which they were resistant (i.e., MAC+AMP or MAC+TET) and were confirmed to be *E. coli* on the basis of lactose utilization, methyl red reaction, and β-galactosidase and β-glucuronidase activities. Additionally, all Amp^r *E. coli* from MAC+AMP plates that were analyzed by PFGE were also tested for resistance to tetracycline by plating on MAC+TET medium.

PFGE. Amp^r and Tet^r *E. coli* from MAC+AMP and MAC+TET plates associated with each dietary treatment (A44, AS700, or control) were chosen for analysis by PFGE. Isolates were randomly selected from fecal deposits across six time points representing short (days 0 and 7), moderate (days 56 and 70), or long (days 126 and 175) periods of environmental exposure and included isolates from each pen. A total of 161 Amp^r (A44, $n = 56$; AS700, $n = 56$; control, $n = 49$) and 237 Tet^r (A44, $n = 79$; AS700, $n = 83$; control, $n = 75$) *E. coli* isolates were selected for PFGE.

Isolates were subtyped via XbaI-digested genomic DNA according to the Centers for Disease Control and Prevention (13) 1-day standardized laboratory protocol for molecular subtyping of *E. coli*. Electrophoresis was performed with

a Chef DR II electrophoresis unit (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Gels were documented using an AlphaImager imaging system (Alpha Innotech, San Leandro, CA). *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. Each time an *E. coli* isolate was cultured for PFGE, one to three plated colonies were suspended in 40 μl of 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA; pH 8.0) and stored at −80°C for PCR analysis of resistance gene determinants.

Resistance determinants encoded by *E. coli*. *E. coli* isolates analyzed by PFGE were tested by PCR for select genes encoding tetracycline (*tetA*, *tetB*, *tetC*, *tetL*, *tetM*, *tetO*, and *tetW*), ampicillin (*bla*$_{OXA1}$, *bla*$_{PSE1}$, and *bla*$_{TEM1}$), and sulfonamide (*sul1* and *sul2*) resistance. Primers and annealing temperatures were previously described for *tetA*, *tetC*, *tetL*, *tetM*, and *tetO* (37), *tetB* and *tetW* (38), *bla*$_{OXA1}$ and *bla*$_{PSE1}$ (22), *bla*$_{TEM1}$ (6), and *sul1* and *sul2* (8). PCR for the detection of each gene was carried out individually. Each PCR mixture (20 μl) contained (final concentrations) 1× HotStarTaq Plus master mix (Qiagen Inc., Mississauga, Ontario, Canada) and 0.4 μM of each primer.

Colonies of *E. coli* were heat lysed (98°C for 4 min) and centrifuged (16,000 × g; 4 min), and the supernatant (1 μl) was added to the PCR mixture as a DNA template. The PCR conditions are as follows: 95°C for 5 min; 35 cycles of 95°C for 20 s, respective annealing temperatures for 30 s, and 72°C for 1 min; and 72°C for 10 min. The PCRs were carried out using an Eppendorf MasterCycler thermal cycler (Eppendorf, Mississauga, Ontario, Canada). Twenty microliters of product was visualized on a 1.5% (wt/vol) agarose gel following electrophoresis and staining with ethidium bromide. All PCRs were run with positive controls (plasmids with sequence-verified genes) for respective gene determinants and negative controls containing water in place of a DNA template.

Statistical analysis. PFGE patterns of *E. coli* isolates were separated into two databases based on the selective medium used for isolation (MAC+AMP or MAC+TET). Within each database, PFGE patterns were either classified as unique or grouped into clusters based on ≥90% homology using Dice similarity coefficients with unweighted pair group methods with arithmetic average algorithms built into Bionumerics software (Bionumerics 5.1; Applied Maths BVBA, Sim-Martens-Latem, Belgium). The number of grouped clusters was used to describe the genetic diversity of *E. coli* from each treatment (A44, AS700, or control) and exposure period (short, moderate, or long), with larger numbers of clusters representing greater diversity. The position tolerance and optimization were set at 1% and 0.5%, respectively.

The Dimensioning Techniques package of the Bionumerics software program was used for analysis of within- and between-group similarities of PFGE groups that were categorized by treatment, duration of exposure within each treatment, and gene profiles of the presence or absence of resistance determinants. For this, binary character profiles using the band-matching option were created after clustering using the Dice coefficient evaluated by the unweighted pair group method. The significance of each group's average within-group similarity was tested using bootstrapping analysis ($n = 1,000$). The null hypothesis was that PFGE profiles within a single group were no more similar than those of a comparison group. The *P* value for genotyping analysis was the percentage of bootstrap resamplings in which the actual within-group similarity was less than that of a randomized group.

E. coli counts were analyzed using the "Proc Mixed" procedure of the SAS software program (42). The pen was considered the experimental unit. The model included the fixed effects of treatment (A44, AS700, or control), time (day of sampling), and the interaction between treatment and time. The repeated statement was applied to the day of sampling, using the pen nested within the treatment as the subject. Various error structures were tested, and the one giving the lowest Akaike information criterion was chosen for analysis. In all instances, *P* values of ≤0.05 were considered significant.

RESULTS

Environmental conditions. The minimum daily air temperature was −14.6°C on day 19, and the maximum was 34.3°C on day 153 (Fig. 1). Daily precipitation was greatest during the month of June (days 93 to 123). The water content of the fecal deposits appeared to correlate with precipitation trends (Fig. 1). By day 84, the water content of all deposits had declined to less than 10%, compared to an initial content of ca. 80% at the start of the experiment. On day 98, the moisture content of deposits increased as a result of the June rainfall.
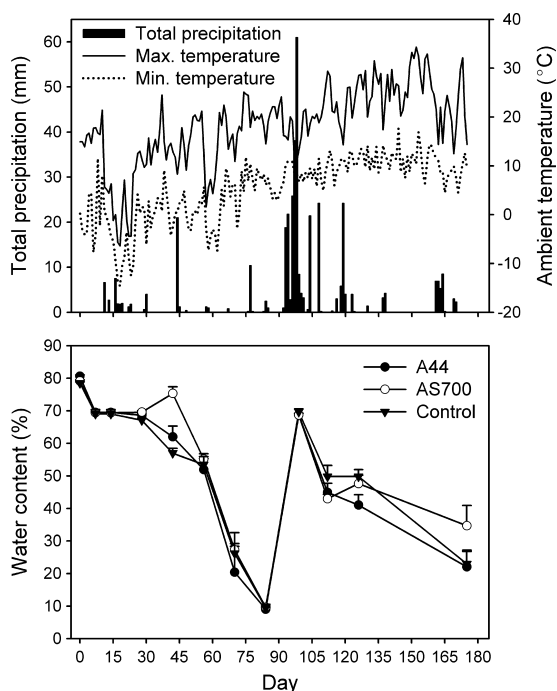
FIG. 1. Daily total precipitation, ambient minimum and maximum air temperatures (top), and mean ($n = 3$; plus standard error) water content of fecal deposits from each treatment group (bottom) over the 175-day experimental period. The treatments were as follows: control, no antimicrobial agents added to the diets of steers from which fecal deposits originated; A44, chlortetracycline (44 ppm); AS700, chlortetracycline and sulfamethazine (each at 44 ppm).
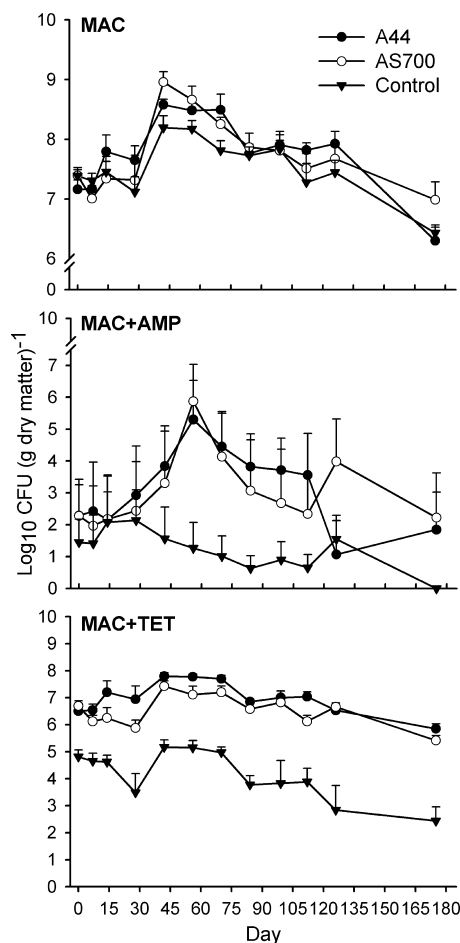

FIG. 2. Mean counts ($n = 3$; plus standard error) of total and ampicillin- and tetracycline-resistant *E. coli* isolated from fecal deposits by using MacConkey agar (MAC) or MAC amended with ampicillin (MAC+AMP, 32 μg ml^{-1}) or tetracycline (MAC+TET, 16 μg ml^{-1}), respectively. The treatments were as follows: control, no antimicrobial agents added to the diets of steers from which fecal deposits originated; A44, chlortetracycline (44 ppm); AS700, chlortetracycline and sulfamethazine (each at 44 ppm).

***Escherichia coli* persistence in artificial fecal deposits.** Generic *E. coli* counts were similar among treatments (Fig. 2, MAC). Only time affected total counts of *E. coli* ($P < 0.001$), with a 1- to 2-log increase in numbers between day 0 and day 42. Numbers of total *E. coli* isolates remained greater than day 0 levels up to day 98 ($P < 0.05$) and decreased ($P < 0.001$) below starting counts only on day 175.

An interaction between time and treatment ($P < 0.001$) was observed for the numbers and persistence of Amp^r *E. coli* in deposits, with numbers being higher and persistence longer for deposits from A44 and AS700 treatments than for the control deposits (Fig. 2, MAC+AMP). Throughout the study, Amp^r *E. coli* accounted for less than 1% of the total generic *E. coli* population. On day 56, Amp^r *E. coli* populations peaked in A44 and AS700 treatment deposits and accounted for 0.07% and 0.16%, respectively, of the *E. coli* population. In contrast, Amp^r *E. coli* numbers in the control treatment exhibited a gradual decline over the experiment, and Amp^r *E. coli* was not detected in control fecal deposits on day 175.

Tet^r *E. coli* in control fecal deposits did not exceed more than 0.3% of the total population but represented from 4.1% (day 126) to 35.6% (day 175) of total *E. coli* in A44 fecal deposits. Similarly, 2.8% (day 56) to 20.2% (day 0) of the *E. coli* in AS700 fecal deposits were Tet^r. An interaction between time and treatment ($P < 0.005$) was also observed for Tet^r *E. coli* (Fig. 2, MAC+TET). Generally, the numbers of Tet^r *E. coli* bacteria in fecal deposits from the controls were less ($P < 0.05$) than those from the A44 and AS700 deposits. In contrast,

counts of Tet^r *E. coli* were similar between A44 and AS700 fecal deposits across time. In control and AS700 fecal deposits, levels of Tet^r *E. coli* declined between days 0 and 28 and then increased and remained greater than day 28 levels for the next three samplings (days 42, 56, and 70). At day 175, the number of Tet^r *E. coli* in control and AS700 fecal deposits declined ($P < 0.05$) to levels below those on day 0. Tet^r *E. coli* levels from A44 fecal deposits initially increased and were greater ($P < 0.05$) on days 42 and 56 than on day 0. Thereafter, levels of Tet^r *E. coli* in the A44 fecal deposits declined, but after 175 days the counts were still not different ($P > 0.05$) from those on day 0 [6.6 versus 5.9 $\log_{10}$ CFU (g DM)$^{-1}$].

Genetic diversity of Amp^r and Tet^r *Escherichia coli*. PFGE profiles were analyzed by using the Dice similarity index to assess the genetic diversity among Amp^r and Tet^r *E. coli* isolates in fecal deposits across treatments and durations of environmental exposure. A total of 13 clusters of Amp^r isolates having $\geq$90% similarity were identified, while eight isolates had PFGE patterns considered unique (Table 1). In general,

TABLE 1. Temporal PFGE clusters (≥90% relatedness) of ampicillin-resistant *E. coli* isolated from fecal deposits of steers fed antimicrobial agents[a]

PFGE cluster group	No. (%) of isolates within antimicrobial treatment and time interval[b]											
	A44				AS700				Control			
	Short	Moderate	Long	Overall	Short	Moderate	Long	Overall	Short	Moderate	Long	Overall
C1A	1 (5.0)			1 (1.8)					1 (5.3)			1 (2.0)
C2A											2 (25.0)	2 (4.1)
C3A	18 (90.0)	6 (33.3)	12 (66.7)	36 (64.3)		3 (14.3)		3 (5.4)		4 (18.2)		4 (8.2)
C4A		1 (5.6)				1 (4.8)		1 (1.8)				
C5A			2 (11.1)	2 (3.6)							4 (50.0)	4 (8.2)
C6A							2 (13.3)	2 (3.6)				
C7A											2 (25.0)	2 (4.1)
C8A									17 (89.5)	18 (81.8)		35 (71.4)
C9A			4 (22.2)	4 (7.1)								
C10A	1 (5.0)	8 (44.4)		9 (16.1)	18 (90.0)	8 (38.1)	10 (66.7)	36 (64.3)				
C11A						6 (28.6)		6 (10.7)				
C12A		2 (11.1)		2 (3.6)								
C13A					2 (10.0)			2 (3.6)				
Unique[c]		1 (5.6)		1 (1.8)		3 (14.3)	3 (20.0)	6 (10.7)	1 (5.3)			1 (2.0)
Total	20	18	18	56	20	21	15	56	19	22	8	49

[a] The degree of relatedness was calculated by using the Dice similarity index of PFGE profiles from isolates across all treatments and time points. *E. coli* was isolated from MacConkey agar amended with ampicillin (32 µg ml⁻¹).

[b] The treatments were as follows: control, no antimicrobial agents added to the diets of steers from which fecal pats originated; A44, chlortetracycline (44 ppm); AS700, chlortetracycline and sulfamethazine (each at 44 ppm). Each time interval represents the duration of environmental exposure of fecal deposits prior to sampling: short, days 0 and 7; moderate, days 56 and 70; long, days 126 and 175. "Overall" represents the genotype across all time intervals within each treatment.

[c] *E. coli* PFGE patterns categorized as unique did not show ≥90% homology to any other isolate patterns.

Amp[r] isolates tended to cluster by treatment. Thirty-five isolates (71.4%) from the control deposits grouped into C8A. Isolates falling into this cluster were present only at the short and moderate exposure periods, accounting for more than 80% of the genetic background, and were exclusive to control deposits. Clusters C2A, C5A, and C7A were isolated only after long exposure, with C2A and C7A being exclusive to control deposits. Most Amp[r] isolates from the A44 and AS700 fecal deposits grouped into one of two major clusters, C3A and C10A, which persisted as the dominant genotypes from early to long exposure intervals. Clusters C5A, C9A, and C12A were exclusive to A44 deposits, whereas C6A, C11A, and C13A were exclusive to AS700 deposits. In A44 and AS700 treatments, Amp[r] isolates from some clusters were collected solely during a single exposure period.

Compared to the Amp[r] isolates, there was greater strain diversity among the Tet[r] isolates (Table 2). In total, 22 clusters of Tet[r] isolates having ≥90% similarity and 8 unique PFGE patterns were identified. As observed with Amp[r] isolates, Tet[r] isolates also tended to cluster by treatment. Tet[r] *E. coli* from control deposits grouped into nine different clusters, and two isolates were unique. Most control Tet[r] isolates (*n* = 40 [53.3%]) grouped into C7T, while no Tet[r] isolates from A44 or AS700 were associated with this cluster. Cluster C7T persisted throughout the experiment, representing 57.1%, 70.6%, and 34.8% of the control genotypes at short, moderate, and long exposures, respectively. Tet[r] isolates from A44 deposits clustered into nine groups, and six isolates had unique genetic profiles. Clusters C8T, C13T, and C20T accounted for most of the A44 Tet[r] isolates and were obtained at all exposure periods. Clusters C6T and C8T accounted for the majority (67.4% total) of Tet[r] *E. coli* from AS700 deposits and were also present at all exposure periods.

Impact of treatment and exposure time on genetic relatedness of Amp[r] and Tet[r] *E. coli*. PFGE patterns of Amp[r] and Tet[r] *E. coli* isolates from each exposure period were compared within treatments to assess the genetic similarity within and between groups (Table 3). Amp[r] *E. coli* isolated at short and moderate exposure times from control deposits showed a high (*P* < 0.001) level of within-group similarity (81.2 and 89.1%, respectively). The high degree of similarity between isolates with short and moderate exposures (89.9%) was supported by the fact that the majority of these isolates fell into C8A (Table 1). This indicated that the Amp[r] *E. coli* isolates were closely related when first deposited in control feces and that this population persisted for moderate periods of exposure but diversified with prolonged periods of exposure (long exposure, 52.6% similarity to short exposure). The within-group diversity of Amp[r] isolates (56.5%) also increased (*P* < 0.001) with the long exposure interval. As with the control, Amp[r] *E. coli* isolates from AS700 deposits were closely (*P* < 0.001) related after a short exposure time (within-group similarity of 82.5%), with this population becoming more diverse after a moderate exposure period (within-group similarity of 59.1%) and remaining so even after long exposure (within-group similarity, 66.1%). The degree of similarity of Amp[r] isolates collected from AS700 deposits after moderate and long exposure periods remained relatively constant at 70.6 and 70.2%, respectively. As with AS700, Amp[r] *E. coli* isolates from A44 deposits were highly (*P* < 0.001) related after short exposure periods (within-group similarity of 87.9%) but diversified after moderate exposure periods (59.4%). After prolonged exposure, the within group similarity of Amp[r] isolates from A44 increased (74.3%; *P* < 0.05). This pattern was also reflected in Amp[r] isolates collected after long exposure, in that they also exhib-

TABLE 2. Temporal PFGE clusters (≥90% relatedness) of tetracycline-resistant *E. coli* isolated from fecal deposits of steers fed antimicrobial agents[a]

PFGE cluster group	A44				AS700				Control			
	Short	Moderate	Long	Overall	Short	Moderate	Long	Overall	Short	Moderate	Long	Overall
C1T									6 (17.1)	2 (11.8)	4 (17.4)	12 (16.0)
C2T									2 (5.7)		2 (8.7)	4 (5.3)
C3T									3 (8.6)			3 (4.0)
C4T					1 (3.2)	3 (17.6)	2 (5.7)	6 (7.2)				
C5T										2 (11.8)	2 (8.7)	4 (5.3)
C6T					11 (35.5)	6 (35.3)	11 (31.4)	28 (33.7)			3 (13.0)	3 (4.0)
C7T									20 (57.1)	12 (70.6)	8 (34.8)	40 (53.3)
C8T	6 (20.7)	5 (27.8)	8 (25.0)	19 (24.1)	4 (12.9)	6 (35.3)	18 (51.4)	28 (33.7)				
C9T					2 (6.5)			2 (2.4)				
C10T					4 (12.9)		1 (2.9)	5 (6.0)				
C11T					8 (25.8)			8 (9.6)		1 (5.9)		1 (1.3)
C12T					1 (3.2)			1 (1.2)			3 (13.0)	3 (4.0)
C13T	5 (17.2)	4 (22.2)	9 (28.1)	18 (22.8)		2 (11.8)		2 (2.4)				
C14T	2 (6.9)			2 (2.5)								
C15T	2 (6.9)	1 (5.6)	1 (3.1)	4 (5.1)								
C16T	2 (6.9)			2 (2.5)								
C17T							3 (8.6)	3 (3.6)				
C18T	5 (17.2)			5 (6.3)								
C19T		2 (11.1)		2 (2.5)								
C20T	5 (17.2)	1 (5.6)	12 (37.5)	18 (22.8)								
C21T		3 (16.7)		3 (3.8)								
C22T									3 (8.6)			3 (4.0)
Unique[c]	2 (6.9)	2 (11.1)	2 (6.3)	6 (7.6)					1 (2.9)		1 (4.3)	2 (2.7)
Total	29	18	32	79	31	17	35	83	35	17	23	75

[a] The degree of relatedness was calculated by using the Dice similarity index of PFGE profiles from isolates across all treatments and time points. *E. coli* was isolated on MacConkey agar amended with tetracycline (16 μg ml^{-1}).

[b] The treatments were as follows: control, no antimicrobial agents added to the diets of steers from which fecal deposits originated; A44, chlortetracycline (44 ppm); AS700, chlortetracycline and sulfamethazine (each at 44 ppm). Each time interval represents the duration of environmental exposure of fecal deposits prior to sampling: short, days 0 and 7; moderate, days 56 and 70; long, days 126 and 175. "Overall" represents the genotype across all time intervals, within each treatment group.

[c] *E. coli* PFGE patterns categorized as unique did not show ≥90% homology to any other isolate patterns.

ited a high degree of relatedness (82.1% similarity) to those that were collected after a short exposure period.

In general, Tet^r isolates exhibited a higher diversity than Amp^r isolates (Table 3), a result that reflects the greater number of clusters identified within this group (Table 2). At early exposure periods, only Tet^r isolates from the AS700 deposits were a distinct population ($P < 0.001$) (within-group similarity of 59.5%). Within-group similarity of Tet^r isolates from AS700

TABLE 3. Degrees of similarity of PFGE group patterns of resistant *E. coli* isolated from fecal deposits of steers as affected by duration of environmental exposure

Resistance and exposure interval group[b]	A44				AS700				Control			
	No. of isolates	Similarity to "short" group	Within-group similarity	P value[c]	No. of isolates	Similarity to "short" group	Within-group similarity	P value	No. of isolates	Similarity to "short" group	Within-group similarity	P value
Ampicillin												
Short	20		87.9	<0.001	20		82.5	<0.001	19		81.2	<0.001
Moderate	18	60.5	58.4	0.999	21	70.6	59.1	0.759	22	89.9	89.1	<0.001
Long	18	82.1	74.3	0.050	15	70.2	66.1	0.994	8	52.6	56.5	<0.001
Tetracycline												
Short	29		61.1	0.979	31		59.5	<0.001	35		66.1	0.101
Moderate	18	54.6	57.2	0.998	17	56.3	60.8	0.19	17	67.8	73.7	<0.001
Long	32	61.0	70.4	<0.001	35	53.7	59.1	0.002	23	63.6	61.1	0.868

[a] Control, no antimicrobial agents added to the diets of steers from which fecal deposits originated; A44, chlortetracycline (44 ppm); AS700, chlortetracycline and sulfamethazine (each at 44 ppm).

[b] Each interval represents the duration of environmental exposure of fecal deposits prior to sampling: short, days 0 and 7; moderate, days 56 and 70; long, days 126 and 175. Ampicillin- or tetracycline-resistant *E. coli* was isolated from MacConkey agar amended with ampicillin (32 μg ml^{-1}) or tetracycline (16 μg ml^{-1}), respectively. The groups of resistant *E. coli* were analyzed separately.

[c] The effect of the exposure interval on the significance of within-group similarity (*P* value) was analyzed within each antimicrobial treatment group. PFGE profiles were grouped by short, moderate, and long exposure intervals and analyzed by bootstrapping ($n = 1,000$).

TABLE 4. Patterns of encoded genetic determinants in ampicillin-resistant[a] *E. coli* strains isolated from fecal deposits of steers fed antimicrobial agents

Gene profile	Detection of gene						No. (%) of isolates within treatment group[b]			
	tetA	*tetB*	*tetC*	*sul1*	*sul2*	bla_{TEM1}	A44	AS700	Control	Overall
G1A	+	−	−	−	−	+	3 (5.4)	0 (0)	5 (10.4)	8 (5.0)
G2A	+	−	−	+	−	+	34 (60.7)	7 (12.5)	0 (0)	41 (25.6)
G3A	+	−	−	−	+	+	0 (0)	0 (0)	1 (2.1)	1 (0.6)
G4A	+	−	+	+	−	+	0 (0)	1 (1.8)	0 (0)	1 (0.6)
G5A	+	−	−	+	+	+	5 (8.9)	0 (0)	0 (0)	5 (3.1)
G6A	−	+	−	−	+	−	0 (0)	3 (5.4)	7 (14.6)	10 (6.3)
G7A	−	+	−	+	+	−	0 (0)	1 (1.8)	0 (0)	1 (0.6)
G8A	−	−	+	−	−	−	9 (16.1)	38 (67.9)	9 (18.4)	56 (34.8)
G9A	−	−	+	−	+	−	1 (1.8)	3 (5.4)	0 (0)	4 (2.5)
G10A	−	−	−	+	−	−	0 (0)	0 (0)	1 (2.1)	1 (0.6)
G11A	−	−	−	−	+	−	1 (1.8)	1 (1.8)	0 (0)	2 (1.3)
G12A	−	−	−	−	−	+	2 (3.6)	0 (0)	5 (10.4)	7 (4.4)
G13A	−	−	−	−	−	−	1 (1.8)	2 (3.6)	21 (43.8)	24 (15.0)
Total							56	56	49	161

[a] *E. coli* was isolated using MacConkey agar amended with ampicillin (32 μg ml^{-1}), and fecal material was sampled from deposits on six occasions over 175 days, representing short, moderate, and long periods of environmental exposure.
[b] Control, no antimicrobial agents added to the diet; the A44 and AS700 antimicrobial feeding treatments are defined in Materials and Methods.

fecal deposits remained relatively constant over the exposure period and was still significant ($P < 0.01$) after long exposure (59.1%). Within-group similarity of Tet^r isolates from A44 deposits increased ($P < 0.001$) after prolonged periods of exposure (70.4%). Similarity of Tet^r isolates collected after moderate and long exposure periods to those collected after short exposure decreased in control deposits. In contrast, Tet^r isolates collected from A44 deposits after long exposure became more similar to those collected during the period of short exposure.

Analysis of isolates across all exposure periods demonstrated that all Amp^r and Tet^r isolates within treatment groups exhibited a high ($P < 0.001$) degree of relatedness. Analysis of exposure periods across all treatments indicated that Amp^r isolates within short and moderate exposure periods were closely ($P < 0.001$) related, whereas this characteristic was observed only in Tet^r isolates collected after long exposure.

Encoded resistance determinants. The gene determinants *tetL*, *tetM*, *tetO*, *tetW*, bla_{OXA1}, and bla_{PSE1} were not detected in any of the isolates. Most of the Amp^r isolates (Table 4) had gene profile G8A (34.8%; positive for *tetC*), followed by profile 2A (25.6%; positive for *tetA*, *sul1*, and bla_{TEM1}). Ninety-eight (60.9%) of the Amp^r isolates did not test positive for bla_{TEM1}, indicating that most of the Amp^r *E. coli* isolates in the study were resistant by a mechanism other than that associated with the Amp^r genes screened. Tet^r genes were detected in 79.1% of Amp^r isolates. *tetC* (37.3%) was most commonly detected, followed by *tetA* (34.3%) and *tetB* (6.9%). In every instance in which an isolate was positive for *tetA*, the same isolate was also positive for *tem1*. Because of the high frequency of Tet^r genes detected in Amp^r *E. coli*, these bacteria were also tested for phenotypic resistance to tetracycline. Each isolate from the A44 and AS700 fecal deposits that encoded a Tet^r gene was resistant to tetracycline, excepting the following: from A44, two isolates and one isolate within the gene profiles G2A and G8A, respectively; from AS700, two isolates within gene profile G8A. Every isolate from control deposits that encoded a Tet^r gene was resistant to tetracycline. Additionally, three and five control isolates within G12A and G13A, respectively, were

resistant to tetracycline despite testing negative for any Tet^r genes (data not shown).

Sulfonamide-resistant genes occurred in 41.2% of Amp^r *E. coli* isolates. *sul1* and *sul2* were detected separately in 26.8% and 10.7% of the Amp^r isolates, respectively, and together in only 3.7% of isolates. Of the Amp^r isolates from A44 deposits, 41 (73.2%) possessed one or both *sul* genes, which was more frequent than the occurrence among Amp^r isolates collected from AS700 fecal deposits (26.8%).

Overall, 12 patterns of encoded resistance genes were detected among the Tet^r isolates (Table 5). The majority of isolates from all treatments possessed profile G6T (overall, 62.9%; positive for *tetB* and *sul2*). G3T was the second most common (13.3%; positive for *tetA*, *sul1*, and *tem1*), and almost all G3T isolates originated from the A44 deposits. Profiles G9T and G10T were detected only in Tet^r *E. coli* from AS700 deposits. Six other profiles (G1T, G2T, G4T, G7T, G8T, and G11T) were also specific to a single treatment. Of the Tet^r isolates examined, 96.3% possessed one or more of the *tetA*, *tetB*, and *tetC* determinants. The most common Tet^r gene was *tetB* (68.5%), followed by *tetA* (13.7%), *tetC* (12.0%), and the combination of *tetA* and *tetB* (1.7%) or *tetB* and *tetC* (0.4%). Most of the Tet^r *E. coli* isolates with *TetC* were from AS700 deposits.

Sulfonamide resistance genes were detected in 87.1% of Tet^r isolates, with *sul2* (72.3%) being the most prevalent, followed by *sul1* (13.3%) and the combination of *sul1* and *sul2* (1.7%). *sul* determinants were detected in 96.2%, 80.7%, and 84.2% of Tet^r *E. coli* isolates from A44, AS700, and control deposits, respectively. Approximately 15% of Tet^r isolates possessed bla_{TEM1}, and all, except one isolate, were from A44 deposits. Only in one instance was *tetA* detected in Tet^r isolates without being associated with bla_{TEM1}.

DISCUSSION

Persistence of *E. coli* in fecal deposits. The present study examined the persistence and diversity of AR *E. coli* in fecal deposits from cattle fed one of three AGP treatments. *E. coli*

TABLE 5. Patterns of encoded genetic determinants in tetracycline-resistant[a] *E. coli* isolated from fecal deposits of steers fed antimicrobial agents

Gene profile	Detection of gene						No. (%) of isolates within treatment group[b]			
	tetA	*tetB*	*tetC*	*sul1*	*sul2*	bla_{TEM1}	A44	AS700	Control	Overall
G1T	+	−	−	−	−	+	1 (1.3)	0 (0)	0 (0)	1 (0.4)
G2T	+	+	−	−	+	−	0 (0)	0 (0)	1 (1.4)	1 (0.4)
G3T	+	−	−	+	−	+	30 (38.0)	1 (1.2)	0 (0)	31 (13.3)
G4T	+	+	−	+	+	+	3 (3.8)	0 (0)	0 (0)	3 (1.3)
G5T	−	+	−	−	−	−	0 (0)	2 (2.4)	10 (14.1)	12 (5.2)
G6T	−	+	−	−	+	−	41 (51.9)	48 (57.8)	60 (80.0)	149 (62.9)
G7T	−	+	+	−	+	−	0 (0)	0 (0)	1 (1.4)	1 (0.4)
G8T	−	+	−	+	+	−	0 (0)	0 (0)	1 (1.4)	1 (0.4)
G9T	−	−	+	−	−	−	0 (0)	10 (12.0)	0 (0)	10 (4.3)
G10T	−	−	+	−	+	−	0 (0)	18 (21.7)	0 (0)	18 (7.7)
G11T	−	−	−	−	+	−	2 (2.5)	0 (0)	0 (0)	2 (0.9)
G12T	−	−	−	−	−	−	2 (2.5)	4 (4.8)	1 (1.4)	7 (3.0)
Total							79	83	75	237

[a] *E. coli* was isolated using MacConkey agar amended with tetracycline (16 μg ml^{-1}), and fecal material was sampled from deposits on six occasions over 175 days, representing short, moderate, and long periods of environmental exposure.

[b] Control, no antimicrobial agents added to the diet; the A44 and AS700 antimicrobial feeding treatments are defined in Materials and Methods.

was isolated using selective plates, a method that has been described previously (24, 43).

Bacteria from fecal material can be transferred in runoff water, potentially contaminating water sources for humans (45). Our work suggests that feces could pose a risk of contaminating watersheds with AR *E. coli* for months after defecation. The fecal deposits presented a matrix that enabled growth and prolonged survival of AR *E. coli* beyond 175 days. Except for Amp^r *E. coli* in control deposits, *E. coli* numbers increased after defecation. This suggests that the initial load of total and resistant *E. coli* deposited in feces may underestimate populations that develop after feces enter the environment. Other studies have also reported a growth phase of *E. coli* in fecal deposits, although most have shown that growth occurred within 21 days of incubation (36, 48). Growth of *E. coli* in fecal material is related to temperature, with a proposed optimal range between 20 and 35°C (48, 56). The delayed growth in our study may have resulted from the low environmental temperatures at the beginning of the experiment, given that growth of *E. coli* occurred when the maximum daily temperature ranged between 14°C and 21°C. It is also possible that *E. coli* growth was partially attributable to reductions in competing bacteria as oxygen penetrated the feces with time (34). This may have reduced numbers of obligate anaerobes, perhaps changing the niche to one more favorable for *E. coli*.

Declines the in *E. coli* population corresponded with a loss of moisture from fecal deposits. However, generic *E. coli* numbers still exceeded 10^7 (g dry matter)$^{-1}$ even when the moisture content of the deposits declined below 10% after 84 days of exposure. Sinton et al. (48) showed that rehydration of fecal deposits promoted regrowth of enterococci and reduced the rate of decline of viable *E. coli*, although growth was more prolific in fecal deposits that contained more than 80% water. In our study, heavy precipitation occurred in June (days 93 to 123). Although this event did not dramatically stimulate the growth of *E. coli*, it did appear to reduce the rate of decline in viable *E. coli* and may have contributed to persistence. It is also

possible that by this time, nutrients as opposed to moisture limited growth.

Although cattle from the control treatment were not fed antimicrobials, their fecal deposits had relatively high numbers of Tet^r *E. coli* on day 0. Similar results have been described for fecal samples that have been collected directly from the rectums of feedlot cattle (1, 46) and dairy calves (25) that have not been fed AGP. Given the widespread prevalence of tetracycline resistance (40), presumably these cattle were colonized by Tet^r bacteria from other environmental sources, most likely prior to their arrival at the feedlot. The fact that Tet^r *E. coli* can survive for up to 175 days in fecal deposits suggests that fecal matter on pasture, surface water sources, herd cohorts, or possibly even wildlife could have served as acquisition sources of Tet^r *E. coli* for cattle prior to their arrival at the feedlot.

As expected, the numbers of Tet^r *E. coli* in fecal deposits from cattle fed AGP containing chlortetracycline were greater than those of Tet^r *E. coli* in fecal deposits from cattle fed no AGP. The AGP affected the concentrations of Tet^r *E. coli* detected in A44 and AS700 fecal deposits to the same extent. This was likely because A44 and AS700 contained the same levels of chlortetracycline (44 ppm), although the AS700 mixture also contained sulfamethazine (44 ppm). Generally, Tet^r *E. coli* from all treatments displayed similar trends, with an initial growth followed by a death phase. Approximately 75% of chlortetracycline administered in the diets of cattle is excreted in feces and urine (29). Elmund et al. (18) reported that levels of chlortetracycline decreased from 14 ppm in fresh bovine feces to 0.34 ppm in aged feces. However, degradation of chlortetracycline is temperature dependent (19). Over 30 days, Gavalchin and Katz (19) showed that soil amended with manure at 4°C, 20°C, or 30°C resulted in 0%, 12%, or 56% degradation of chlortetracycline, respectively. These data would suggest that chlortetracycline concentrations would have been greater in fecal deposits early in our experiment when temperatures were low, subsequently degrading more rapidly when temperatures increased at later time points.

Throughout the entire course of our study, the percentage of Tet^r *E. coli* in the total population remained relatively constant in fecal deposits regardless of treatment. Consequently, it appears that the selective pressure of subtherapeutic tetracycline was more potent for Tet^r *E. coli* in the digestive tracts of cattle than in deposited feces. This would also imply that in the absence of selection pressure in control fecal deposits, the fitness of Tet^r *E. coli* was not inhibited by retaining encoded resistance to chlortetracycline. This is in agreement with an earlier study that reported selection and maintenance of *E. coli* with a streptomycin-sulfadiazine-tetracycline-resistant phenotype in dairy cattle that was independent of antimicrobial administration (26). The same authors suggested that resistance elements were maintained because they were linked to additional genetic traits conferring a selective advantage (27). There may also be advantages to *E. coli* maintaining resistance elements in secondary environments.

Although initial Amp^r *E. coli* counts in control deposits were similar to those of A44 and AS700 deposits, numbers of Amp^r *E. coli* in control deposits did not increase significantly after defecation. In contrast, numbers of Amp^r *E. coli* in A44 and AS700 deposits increased dramatically from initial levels. Despite a large standard error of the means, counts of Amp^r *E. coli* from treatment fecal deposits were statistically different and, on day 56, were approximately four log units greater than those for control Amp^r *E. coli*. The reasons for these differences are unclear but may be related to the AGP fed to cattle. The genetic backgrounds of Amp^r *E. coli* in A44 and AS700 fecal deposits were significantly different from those in control deposits. It may be possible that while within the ruminant digestive tract, the AGP selected for Amp^r *E. coli* populations that in some unknown way were better able to persist in secondary environments than were Amp^r *E. coli* populations from cattle that were not administered AGP.

Genetic diversity of *Escherichia coli*. Despite the genetic analysis being limited to only Amp^r and Tet^r *E. coli* isolates, considerable genetic diversity among isolates was observed. Others have also shown high diversity in the *E. coli* populations from cattle (2, 43). Survival of *E. coli* outside the primary habitat of the host's intestinal tract has been hypothesized to be limited and transient as a result of fluctuations in nutrient availability and temperature (58). However, there is evidence that certain strains of *E. coli* are adapted to survive in secondary habitats, such as soil and water (11) and manure storage systems (17). It has been hypothesized that once the bacteria are shed from the digestive tract, a multitude of factors select for fit subpopulations of *E. coli*, leading to an expansion in genetic diversity as strains persist in a variety of environments (21, 53). Our results show that the diversity of Amp^r *E. coli* within treatment groups increased after environmental exposure, even within the relatively restricted habitat of the fecal deposit. The increase in diversity of Amp^r *E. coli* isolated from A44 and AS700 deposits was apparent after only moderate exposure periods. The fact that *E. coli* in feces with a short duration of exposure would have recently emerged from the comparatively steady-state conditions of the digestive tract may have led to a higher degree of relatedness among isolates obtained at this point in the study.

None of the eight Amp^r clusters associated with long-exposed control deposits had been identified previously. Because the fecal deposits were exposed to environmental conditions, it cannot be ruled out that contaminating sources, including cross-contamination from adjacent samples, contributed to the Amp^r *E. coli* populations and the detection of new PFGE clusters after long exposure. Regardless, there was a clear contrast between Amp^r *E. coli* isolates in A44 and AS700 deposits and those from control deposits in that among Amp^r *E. coli* isolates from the treatment deposits, main PFGE cluster groups survived throughout the experiment. This led us to speculate that the Amp^r *E. coli* present initially in control fecal deposits was less fit than that in the A44 or AS700 fecal deposit, although it is difficult to explain why. This speculation may also be relevant to why Amp^r *E. coli* numbers in control fecal deposits did not increase; they may have been outcompeted by other bacteria in the fecal deposits.

Tet^r *E. coli* isolates were genotypically more diverse than Amp^r *E. coli*, a result that is perhaps not surprising given the far higher numbers of Tet^r than Amp^r *E. coli* isolates in deposits. There were numerous instances where distinct PFGE clusters were detected in fecal deposits only after short exposure periods, a result suggesting that there was differential Tet^r *E. coli* survival in deposits after defecation. However, as with Amp^r *E. coli* in A44 and AS700 fecal deposits, the Tet^r *E. coli* PFGE cluster groups that encompassed the majority of *E. coli* within each treatment at the beginning of the experiment were stable across time.

Overall, there was a clear relationship between treatment and the genetic diversity of resistant *E. coli* in fecal deposits. It was interesting that the within-group similarities were highest for Amp^r and Tet^r *E. coli* isolates from control deposits compared to *E. coli* isolates from A44 and AS700 deposits. We hypothesized that the selective pressure of including AGP in the diet would reduce the within-group diversity of *E. coli* by selecting for resistant strains, but in the present experiment this was clearly not the case. The time period of environmental exposure also influenced the genotypic nature of *E. coli* in fecal deposits, perhaps indicating that some genotypes survive better in secondary environments. These findings have implications for microbial source tracking since the nature of *E. coli* released from the point source may change with both diet and time of environmental exposure (21). Knowledge of genetic markers and of the external factors that influence their frequency is important for accurate bacterial source tracking (31).

Resistance determinants encoded by *Escherichia coli*. The only β-lactamase gene detected in *E. coli* in this study was *bla*$_{TEM1}$. This determinant is generally plasmid mediated (9) and is one of the more prevalent β-lactamases (32). Similar to our results, *bla*$_{TEM1}$ has been reported to be the most common β-lactamase gene detected among Amp^r *E. coli* isolates from livestock (9). The genes *tetL*, *tetM*, and *tetW* were not detected in any of the *E. coli* in our study. Generally, these genes are associated with gram-positive bacteria (40), but a recent investigation detected *tetM* and *tetW* in *E. coli* isolated from farm soils (50). These results would suggest that *E. coli* may acquire these more-obscure *tet* determinants through interaction with microbes in the environment as opposed to those in the digestive tract.

Gene profiles were clearly related to treatment. This outcome indicates that the type of AGP administered to cattle had an impact on the nature of Amp^r *E. coli* in deposits. More than

90% of Amp[r] *E. coli* from A44 and AS700 fecal deposits possessed Tet[r] genes, although these populations were still clearly distinct. Almost all of the Amp[r] *E. coli* isolates encoding *tetA* were from A44 deposits, whereas most encoding *tetC* were from AS700 deposits. Presumably, this difference arises from the presence of a mixture of tetracycline and sulfamethazine in AS700 as opposed to tetracycline alone in A44. Sulfamethazine is relatively stable in manure (3, 16) and would likely have been prevalent in AS700 fecal deposits throughout our study. Even though AS700 contained sulfamethazine, a large number of the isolates from these deposits lacked *sul1* or *sul2*. In fact, the majority of Amp[r] isolates that possessed *sul1* and *sul2* were associated with A44 deposits. It is not clear why this occurred.

Except for five isolates, all Amp[r] *E. coli* isolates that possessed *tetA*, *tetB*, or *tetC* were resistant to tetracycline. Tetracycline-susceptible *E. coli* strains that encode resistance genes have been reported previously (14). Additionally, some *E. coli* isolates from control deposits tested negative for tetracycline resistance genes yet were resistant to tetracycline. Likely these *E. coli* isolates encoded a resistance gene not tested in our investigation. The high level of both phenotypic resistance (80.7%) and tetracycline resistance genes (79%) in Amp[r] *E. coli* supports previous research showing a link between tetracycline and additional resistance determinants. Our laboratory and others have previously reported that the feeding of tetracycline-based AGP to cattle can promote multiresistance to ampicillin and tetracycline in *E. coli* (1, 23). In addition, resistance determinants for ampicillin and tetracycline have been identified on a common plasmid (30, 47). These data further highlight the selection of multiresistant *E. coli* when subtherapeutic levels of tetracycline are administered to cattle. Our results show that multiresistant *E. coli* can survive for extended periods in the environment.

The most common gene profile detected in Tet[r] *E. coli* isolates was G6T, which included *tetB* and *sul2*, a result that confirms the possible link between these two determinants as observed for Amp[r] isolates. Similarly, Boerlin et al. (8) found an association between *tetB* and *sul2*. In that same study, an association was also noted between *tetA* and *sul1*. Our results support their conclusion, in that G3T, which included *tetA*, *sul1*, and *bla*_{TEM1}, was the second most prevalent profile in Tet[r] *E. coli*. Only in a single isolate was *tetA* not detected along with *bla*_{TEM1}.

There were clear differences in the resistance genes found in Tet[r] *E. coli* compared to those in Amp[r] *E. coli*. Notably, *tetB* was the most prevalent determinant in Tet[r] *E. coli*. The prevalence of *tetB* in generic *E. coli* from cattle has previously been shown to be higher than that of *tetA* (10, 43). Although *tetB* may be the most common determinant in generic *E. coli*, there clearly are subpopulations of *E. coli* (e.g., Amp[r] *E. coli*) in which other *tet* determinants dominate. Blake and colleagues (7) reported differences in Tet[r] genes in *E. coli* isolated from pigs fed or not fed antimicrobials. We also report differences in Tet[r] genes carried by *E. coli* in feces from cattle fed diets with or without AGP. An interesting finding from our study was that chlortetracycline administered to animals at similar concentrations can select equally for phenotypic resistance (viz., Tet[r] *E. coli* counts) through different genes encoding the resistance. As noted previously, differential selection of resistance genes may alter multiresistance through carriage of linked resistance

determinants (7). A genetic link between *tetA* and class 1 integrons on the same conjugative plasmid has recently been reported (52), which may suggest that selection for *tetA* (largely seen in Amp[r] *E. coli* isolates from A44 deposits) could also select for class 1 integrons. Characterization of the selective pressures exerted by AGP that are responsible for a differential prevalence of resistance determinants could provide additional insight into the ecology of antimicrobial resistance.

In conclusion, bovine fecal deposits present a matrix for the growth and persistence of Tet[r], Amp[r], and generic *E. coli* beyond 175 days. Resistant *E. coli* populations in fecal material can increase after defecation, increasing the true microbial load introduced into the environment. Inclusion of AGP in the diet promotes the presence and preferential persistence of AR *E. coli* in fecal deposits. However, Tet[r] *E. coli* persisted for 175 days even in fecal deposits that arose from cattle that had not been fed AGP. This suggests that encoding of Tet resistance had minimal effects on environmental fitness, even in the absence of antimicrobials. Genotypic diversity of Amp[r] *E. coli* isolates tended to increase with longer periods of environmental exposure, whereas this trend was less apparent for Tet[r] *E. coli*, a result that may reflect the greater initial diversity of Tet[r] isolates. Fecal deposits can serve as a reservoir of AR *E. coli* for months after defecation and should be considered a potential point-source contaminant in the development of watershed management plans.

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