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Molecular Epidemiology of Ceftiofur-Resistant *Escherichia coli* Isolates from Dairy Calves

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Healthy calves ($n = 96$, 1 to 9 weeks old) from a dairy herd in central Pennsylvania were examined each month over a five-month period for fecal shedding of ceftiofur-resistant gram-negative bacteria. Ceftiofur-resistant *Escherichia coli* isolates ($n = 122$) were characterized by antimicrobial resistance (disk diffusion and MIC), serotype, pulsed-field gel electrophoresis subtypes, beta-lactamase genes, and virulence genes. Antibiotic disk diffusion assays showed that the isolates were resistant to ampicillin (100%), ceftiofur (100%), chloramphenicol (94%), florfenicol (93%), gentamicin (89%), spectinomycin (72%), tetracycline (98%), ticarcillin (99%), and ticarcillin-clavulanic acid (99%). All isolates were multidrug resistant and displayed elevated MICs. The *E. coli* isolates belonged to 42 serotypes, of which O8:H25 was the predominant serotype (49.2%). Pulsed-field gel electrophoresis classified the *E. coli* isolates into 27 profiles. Cluster analysis showed that 77 isolates (63.1%) belonged to one unique group. The prevalence of pathogenic *E. coli* was low (8%). A total of 117 ceftiofur-resistant *E. coli* isolates (96%) possessed the bla_{CMY2} gene. Based on phenotypic and genotypic characterization, the ceftiofur-resistant *E. coli* isolates belonged to 59 clonal types. There was no significant relationship between calf age and clonal type. The findings of this study revealed that healthy dairy calves were rapidly colonized by antibiotic-resistant strains of *E. coli* shortly after birth. The high prevalence of multidrug-resistant nonpathogenic *E. coli* in calves could be a significant source of resistance genes to other bacteria that share the same environment.

Antibiotic use for preventing disease and promoting growth of healthy animals is an integral part of livestock production in the United States. Fifty years after the initial approval of antibiotic-medicated feeds for livestock to improve overall health and increase productivity, the use of antibiotics in food-producing animals to maintain current levels of production is met with ever-increasing controversy (1). In the last few years, there have been important changes in perceptions and priorities of federal agencies regarding antibiotic use in animals. The emergence of antibiotic resistance among pathogens has been a growing concern in veterinary medicine (9, 37). Antibiotic-resistant pathogens pose the threat of severe and costly animal health problems. Furthermore, the increasing level of resistance to frontline antimicrobial agents important in treating human diseases, such as expanded-spectrum cephalosporins and fluoroquinolones, is a significant public health concern (1).

Calf mortality and treatment costs represent an enormous economic loss to the dairy industry, estimated to surpass $250 million annually in the United States (35). Numerous studies have shown that the most prevalent causes of death in calves from birth to weaning are diarrhea and respiratory diseases. A recent survey by the USDA National Animal Health Monitoring System reported that diarrhea accounted for 62.1% of the deaths of unweaned calves (24). The extended-spectrum cephalosporin ceftiofur (Cef) has been approved for therapeutic use in cattle in the United States since 1988. Ceftiofur has normally been used for the treatment of respiratory tract infections, metritis, and foot rot (43). The administration of ceftiofur to treat diarrhea in calves constitutes extralabel drug use and requires the approval of the herd veterinarian (10). In the past few years, *Escherichia coli* strains resistant to multiple, structurally unrelated drugs, including newer antibiotics used to treat severe cases of calf scours, such as aminoglycosides, fluoroquinolones, and expanded-spectrum cephalosporins, have been found in diarrheic calves (7, 41, 42). The emergence of such multidrug-resistant pathogenic strains of *E. coli* poses an increasing threat to the successful management of calf scours.

A recent study conducted by Sawant et al. (33) on antibiotic usage in Pennsylvania dairy herds showed that tetracycline (Tet), neomycin, and spectinomycin (Spt) were the most commonly used antibiotics in the treatment of enteritis in calves. In that study, a total of 113 dairy producers were surveyed on their antibiotic use practices. Producers of 6 of the 113 dairy herds volunteered for an ongoing study on the effects of tetracycline use on antimicrobial resistance in their herds. They were surveyed for 12 months on the prevalence and distribution of antibiotic-resistant gram-negative enteric bacteria (GNEB) in fecal samples from lactating cows and calves. The calves on one farm shed higher levels (88.5%) of ceftiofur-resistant (Cef^r) GNEB than calves on the other five farms (0 to 1%). This observation was intriguing, and it was decided that conducting a more intensive study on the herd with high levels of ceftiofur resistance would help to decipher the molecular epidemiology of ceftiofur resistance of GNEB in dairy herds. The objective of this study was to understand and elucidate the molecular epidemiology of ceftiofur-resistant GNEB shed by

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healthy dairy heifer calves. It is anticipated that this further characterization of ceftiofur-resistant organisms will advance the knowledge of antibiotic-resistant GNEB isolated from healthy calves.

MATERIALS AND METHODS

Herd survey. Fecal samples from calves (1 to 9 weeks of age) on a 600-cow Holstein dairy farm in central Pennsylvania were collected for bacteriological analysis. All calves born from April through August 2003 were included in the sampling population for the study. Over the course of the five-month study, 96 calves were sampled. The farm reported the use of antibiotics, including ceftiofur sodium (Naxcel; Pharmacia & Upjohn Company, Kalamazoo, MI), to treat scours and respiratory infections in calves. However, the farm did not keep records of the antibiotic treatment for each animal. Management practices on this farm include raising heifer calves born on the farm for replacement stock. Prior to freshening, cows were housed in a community maternity pen in a free-stall barn, and cows were moved to individual calving pens to deliver. The free-stall barn had sand bedding, but the individual pens had straw. Calves remained with their dams for several hours and were fed 6 quarts of colostrum in addition to nursing on the first day. On the farm, preweanling- and weanling-aged calves were housed in individual pens with straw bedding in open-faced calf sheds; the sheds were located in an area away from older heifers and adult cows. The pens were separated by solid plywood sheets, but the front opening allowed for nose-to-nose contact between older calves. Calves were fed 4 quarts of milk replacer daily, along with free-choice water in individual buckets. The farm had a history of using tetracycline-neomycin-medicated milk replacers but switched to a nonmedicated milk replacer in May 2003 of the study. Calves were weaned at 8 to 9 weeks of age. Calves were selected for sampling if they met the study criteria, namely, if they were <60 days old and did not display signs of illness or scours.

Bacterial isolation and identification. Fecal samples from 20 dairy heifer calves were collected every 4 weeks for a period of 5 months (April to August 2003). One gram of feces was diluted in 9 ml of 0.85% sterile saline solution. The contents were mixed thoroughly and 10-fold serially diluted. The dilutions were plated on MacConkey agar (MAC) (Oxoid Ltd., United Kingdom) supplemented with ceftiofur at 8 μg/ml (MAC-CEF) (Pharmacia & Upjohn Company, Kalamazoo, MI); a MAC plate without the antibiotic was used as the control plate. This concentration of ceftiofur (8 μg/ml) is recommended for the selection of ceftiofur-resistant bacteria by the NCCLS (now known as the CLSI), based on veterinary interpretative criteria for bovine respiratory disease pathogens (25). Currently, there are no CLSI breakpoints approved to indicate ceftiofur resistance in gram-negative enteric bacteria. The inoculated plates were incubated at 37°C for 24 h. The number of colonies on MAC and MAC-CEF were counted and expressed as total GNEB CFU/g of feces and total Cef^r GNEB CFU/g of feces, respectively. Two to three colonies were selected from each MAC-CEF plate with bacterial growth and subcultured. The isolates were speciated using an API 20E kit (BioMérieux, St. Louis, MO). Isolates that had an identification score of >97% were included in the study.

Antimicrobial resistance testing. Cef^r *E. coli* isolates were screened for antibiotic resistance to ampicillin (Amp) (10 μg/ml), chloramphenicol (Chl) (30 μg/ml), gentamicin (Gen) (10 μg/ml), enrofloxacin (Eno) (5 μg/ml), Tet (30 μg/ml), Spt (100 μg/ml), ticarcillin (Tic) (75 μg/ml), ticarcillin-clavulanic acid (Tim) (75 and 10 μg/ml, respectively) (Remel, Lenexa, KS), ceftiofur (Cef) (30 μg/ml), and florfenicol (Ffc) (30 μg/ml) (Becton Dickinson and Company, Sparks, MD) using a Kirby Bauer disk diffusion assay according to standards and interpretive criteria described by NCCLS (25). Intermediate zones of inhibition were counted as sensitive for purposes of this study. Antimicrobial MICs were determined by the broth microdilution method in cation-adjusted Mueller-Hinton broth (Becton Dickinson and Company, Sparks, MD) as described by NCCLS, including suggested breakpoints to determine resistance and susceptibility (25). Currently, there are no CLSI breakpoints to analyze resistance to ceftiofur, spectinomycin, and florfenicol in *E. coli* of bovine origin; in this study, the resistance breakpoints for bovine respiratory disease pathogens were used. The isolates were screened for antibiotic resistance to Amp, Chl, Spt, and Tet (MP Biomedicals LLC, Aurora, OH) and Cef, Ffc, Gen, Tic, and Tim (Sigma-Aldrich, St. Louis, MO). The MIC was defined as the lowest concentration of antibiotic completely inhibiting visible growth. Two quality control strains, *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA), were included in each assay.

Serotype analysis. Cef^r *E. coli* strains were grown on brain heart infusion agar slants (Oxoid Ltd., United Kingdom) at 37°C for 24 h. The isolates were serotyped for O and H groups at the Pennsylvania State University Gastroenteric Disease Center, University Park, Pa., using the procedures described by Orskov et al. (28). H groups were assigned based on restriction fragment length polymorphism analysis by digesting the amplified *fliC* gene with the HpaII restriction enzyme, as described by Machado et al. (21) with modifications. The profiles were compared to the 52 standard H groups for H type designation.

Pulsed-field gel electrophoresis. The Cef^r *E. coli* isolates (n = 122) were genotyped using the pulsed-field gel electrophoresis (PFGE) technique described by Hegde et al. (14). DNA was digested using the XbaI enzyme (New England BioLabs, Beverley, MA). Electrophoresis was performed using a CHEF Mapper XA PFGE system (Bio-Rad, Hercules, CA). Genotypic relatedness was determined using PFGE DNA fingerprint subtypes. Fingerprint subtypes were analyzed using Gel Doc 2000 Molecular Analyst Fingerprinting Plus software, version 6.1 (Bio-Rad, Hercules, CA). The relatedness of restriction profiling was generated by the unweighted pair group method with arithmetic average clusters based on Dice coefficients of each band pattern.

PCR-based assays. Cef^r *E. coli* isolates were evaluated for the presence of several virulence factors of veterinary significance, including STa, CNF1, CNF2, Stx1, Stx2, CS31A, F1845, and K99 (Table 1). The genes coding for STa, CNF1, CNF2, CS31A, F1845, and K99 were detected using the primers, PCR, and run conditions used by the Gastroenteric Disease Center, University Park, Pa. Identification of Stx-1 and -2 was done as described by Meng et al. (22). The *E. coli* isolates (n = 122) were screened for the presence of the cephamycinase gene bla_{CMY2}. Isolates negative for bla_{CMY2} were screened for other beta-lactamase genes, including $bla_{CTX\text{-}M2}$, bla_{FOX}, bla_{SHV}, bla_{TEM}, and *ampC* (Table 1). Amplification was performed with consensus primers for *ampC*, which recognize any plasmid-borne cephamycinase genes derived from the chromosomal *ampC* gene of *Citrobacter freundii* (44). The PCR assays were performed in a thermocycler (PTC 200 Thermocycler; MJ Research, Watertown, MA). The total reaction volume was 25 μl and consisted of puRETaq Ready-To-Go PCR beads (Amersham Biosciences, Germany), forward and reverse primers, DNA template (5 μl of the bacterial DNA extract), and double-distilled water to the final volume. The PCR cycle conditions were optimized for each primer set. Briefly, the PCR cycling conditions consisted of initial denaturation at 94°C for 5 min, followed by 30 cycles each of denaturation at 94°C for 30 s, annealing at the temperature optimal for each primer set for 45 s, and extension at 72°C for 45 s. The amplified PCR product was electrophoresed on a 2% agarose gel in Tris-acetate-EDTA buffer. A 100-bp DNA ladder (Invitrogen, Carlsbad, CA) was used as a molecular weight marker. Positive and negative controls strains were used throughout the PCR-based assays.

Data analysis. The effects of age and sample date on total GNEB and Cef^r GNEB counts were analyzed using the Kruskal-Wallis test. We used the chi-square test to compare number of samples and clonal type (CT) incidence with respect to sample date and calf age. Statistical analyses were performed with MiniTab for Windows (version 13; State College, PA). Statistical significance was accepted at a P value of <0.05.

RESULTS

Prevalence of Cef^r GNEB. Cef^r GNEB were isolated from 85 of 96 calves sampled (88.5%). Over the course of the study, the percentage of calves that shed Cef^r GNEB by month ranged from 65 to 100% (Table 2). There were no significant differences in the frequencies of isolation of Cef^r GNEB for different months ($P < 0.896$). Total GNEB counts ranged from 2.1×10^4 to 1.31×10^{11} CFU/g (mean, 1.69×10^9 CFU/g). The Cef^r GNEB in feces ranged from 0 to 8.5×10^{10} CFU/g (mean, 9.15×10^8 CFU/g). The Cef^r GNEB accounted for 0 to 65.4% of the total fecal gram-negative enteric organisms. There was a one-log difference between the mean Cef^r GNEB and mean GNEB counts. Mean counts for total GNEB and Cef^r GNEB were compared by month sampled and by animal age (data not shown). The mean GNEB and Cef^r GNEB were highest for the month of June. One-week-old calves had the highest mean GNEB and Cef^r GNEB counts. Over the five-month study, there was a significant relationship between bacterial counts and sample month ($P < 0.001$). Both GNEB and

TABLE 1. PCR primers used in this study

Target	Primer sequence	Amplicon size (bp)	Reference
STa	Forward 5′ TCC GTG AAA CAA CAT GAC GG 3′ Reverse 5′ ATA ACA TCC AGC ACA GGC AG 3′	244	11
CNF-1	Forward 5′ GAA CTT ATT AAG GAT AGT 3′ Reverse 5′ CAT TAT TTA TAA CGC TG 3′	543	11
CNF-2	Forward 5′ AAT CTA ATT AAA GAG AAC 3′ Reverse 5′ CAT GCT TTG TAT ATC TA 3′	543	11
Stx-1	Forward 5′ TGT AAC TGG AAA GGT GGA GTA TAC A 3′ Reverse 5′ GCT ATT CTG AGT CAA CGA AAA ATA AC 3′	210	22
Stx-2	Forward 5′ GTT TTT CTT CGG TAT CCT ATT CC 3′ Reverse 5′ GAT GCA TCT CTG GTC ATT GTA TTA C 3′	484	22
CS31-A	Forward 5′ AAT TAG GGC GGG TAA AGA 3′ Reverse 5′ CAT CAC CAG TAG TCA TCA CC 3′	204	11
F1845	Forward 5′ CAC TGT GGG CTC CGC GCA AGC 3′ Reverse 5′ CGG TGA GGT TCA GTG TGT AT 3′	419	11
K99	Forward 5′ TGG GAC TAC CAA TGC TTC TG 3′ Reverse 5′ TAT CCA CCA TTA GAC GGA GC 3′	450	11
bla$_{CMY2}$	Forward 5′ GAC AGC CTC TTT CTC CAC A 3′ Reverse 5′ TGG AAC GAA GGC TAC GTA 3′	1,000	45
bla$_{SHV}$	Forward 5′ ATG CGT TTA TAT TCG CCT GTG 3′ Reverse 5′ TTA GCG TTG CCA GTG CTC GA 3′	861	19
bla$_{CTX-M2}$	Forward 5′ ATG ATG ACT CAG AGC ATT CG 3′ Reverse 5′ TCA GAA ACC GTG GGT TAC GA 3′	877	19
bla$_{FOX}$	Forward 5′ ATG CAA CAA CGA CGT GCG TTC GCG 3′ Reverse 5′ TCA CTC GGC CAA CTG ACT CAG GAT 3′	1,149	19
bla$_{TEM}$	Forward 5′ ATG AGT ATT CAA CAT TTC CGT G 3′ Reverse 5′ TTA CCA ATG CTT ATT CAG TGA G 3′	861	38
ampC	Forward 5′ ATG ATG AAA AAA TCG TTA TGC 3′ Reverse 5′ TTG CAG CTT TTC AAG AAT GCG C 3′	1,143	44

Cef^r GNEB counts decreased as calf age increased ($P < 0.001$).

Identification of Cef^r GNEB. A total of 136 isolates from 85 calves were speciated using an API 20E identification kit (BioMérieux, Hazelwood, MO). The isolates were identified as *Aeromonas* spp. ($n = 2$), *Burkholderia cepacia* ($n = 1$), *Enterobacter sakazakii* ($n = 2$), *Escherichia coli* ($n = 122$), *Klebsiella* spp. ($n = 3$), *Pseudomonas fluorescens* ($n = 2$), and *Salmonella* spp. ($n = 4$). Cef^r *E. coli* was the predominant organism and was isolated from 79 of 85 calves (93%).

TABLE 2. Prevalence of Cef^r GNEB isolated from dairy calves from April to August 2003

Date	Total no. of calves sampled	No. (%) of calves shedding Cef^r GNEB[a]	Mean calf age (wk)
April 2003	20	13 (65)	2.6
May 2003	20	17 (85)	3.5
June 2003	19	19 (100)	5.4
July 2003	17	17 (100)	3.8
August 2003	20	19 (95)	3.4
Total	96	85 (88.5)	3.7

[a] Test of significance for calves shedding Cef^r GNEB by month, χ^2 (P) = 1.089 (0.896).

Antibiotic susceptibility of Cef^r *E. coli* isolates. An antibiotic disk diffusion assay showed that the isolates were resistant to Amp (100%), Cef (100%), Chl (94%), Ffc (93%), Gen (89%), Spt (72%), Tet (98%), Tic (99%), and Tim (99%). No resistance to Eno was observed. Of the 122 isolates tested, all isolates were resistant to 3 or more antibiotics, with 82 of 122 isolates (67%) being resistant to 9 out of 10 antibiotics (Table 3). Antimicrobial MICs for the Cef^r *E. coli* isolates were determined using a broth microdilution assay using NCCLS standards (25); the range of MICs and the MICs at which 50% and 90% of the tested strains were inhibited (MIC_{50} and MIC_{90}, respectively) are presented in Table 4. MIC results confirmed antibiotic resistance profiles generated by the disk diffusion assay. Elevated MICs of all nine drugs were observed (Table 4). All Cef^r *E. coli* isolates had a MIC of ≥16 μg/ml for ceftiofur. The MIC_{90} values for Chl and Ffc were >256 μg/ml, and the MIC_{90} values for Spt and Tic were >1,024 μg/ml. Amp and Tet each had MIC_{90} values of 512 μg/ml.

O and H serotyping. Serotyping of the Cef^r *E. coli* isolates ($n = 122$) was done at the Pennsylvania State University Gastroenteric Disease Center, University Park, Pa. Cef^r *E. coli* isolates belonged to 16 O serogroups and 28 H serogroups (Table 3). The Cef^r *E. coli* isolates were classified as belonging to 42 different O:H serotypes. Of the 122 *E. coli* isolates, 70 (57.3%) belonged to the O8 serogroup, and 60 isolates (49.2%) were serotyped as O8:H25 (Table 3).

PFGE analysis. The *E. coli* isolates were studied for genetic relatedness by means of PFGE. The 122 isolates belonged to 27 distinct PFGE profiles (PFPs) (Fig. 1). Cluster analysis showed that the 27 PFPs belonged to six clusters. Seventy-seven Cef^r *E. coli* isolates (63.1%) belonged to cluster 4 (PFPs 2, 7, 8, 11, 13, and 14). PFP 7 was the most predominant profile observed, accounting for 58 isolates (47.5%). The isolates belonging to PFP 7 had identical antibiotic resistance profiles (Table 3). The isolates in clusters 1 to 3 belonged to PFPs that were found less frequently. Cluster 6 was the smallest, with only a single PFP.

PCR detection of virulence genes. The Cef^r *E. coli* isolates were screened by PCR assays for the presence of genes encod-

TABLE 3. Characteristics of Cef^r *E. coli* clonal types

Clonal type	Antibiogram	Serotype	PFGE type	Virulence gene product(s)	*bla* gene product(s)	No. of isolates (%)
1	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H$^+$	14		CMY2	1 (0.82)
2	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O$^-$:H10	22		CMY2	1 (0.82)
3	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O106:H23	4		CMY2	1 (0.82)
4	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O106:H23	5		CMY2	4 (3.28)
5	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O106:H49	5		CMY2	1 (0.82)
6	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H11 or O$^-$:H47	17		CMY2	1 (0.82)
7	Amp-Chl-Gen-Spt-Tet-Tic-Tim-Cef	O111:H$^+$	8	Stx-1	CMY2	1 (0.82)
8	Amp-Chl-Ffc-Tet-Tic-Tim-Cef	O119:H$^+$	12	STa, CS31A	CMY2	1 (0.82)
9	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H$^-$	14		CMY2	1 (0.82)
10	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O15:H11 or O145:H11	24		CMY2	1 (0.82)
11	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O150:H1	21		CMY2	1 (0.82)
12	Amp-Tic-Tim-Cef	O$^-$:H19	9	STa	CMY2	1 (0.82)
13	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H20	13		CMY2	1 (0.82)
14	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O$^-$:H21	3		CMY2	1 (0.82)
15	Amp-Chl-Ffc-Tet-Tic-Tim-Cef	O2:H30	27	F1845	CMY2	1 (0.82)
16	Amp-Chl-Spt-Tet-Tic-Tim-Cef	O$^-$:H23	2	F1845	CMY2	1 (0.82)
17	Amp-Chl-Ffc-Tet-Tic-Tim-Cef	O$^-$:H25	23		CMY2	1 (0.82)
18	Amp-Tet-Tic-Tim-Cef	O33:H17	16		CMY2	2 (1.64)
19	Amp-Tet-Tic-Tim-Cef	O33:H4	16		CMY2	1 (0.82)
20	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O3:H36	2		CMY2	1 (0.82)
21	Amp-Chl-Gen-Ffc-Spt-Tic-Tim-Cef	O$^-$:H37	25		CMY2	1 (0.82)
22	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O4:H$^+$	1		CMY2	1 (0.82)
23	Amp-Chl-Ffc-Tet-Tic-Tim-Cef	O$^-$:H4	23		CMY2	2 (1.64)
24	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O4:H30	10	F1845	CMY2	1 (0.82)
25	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O4:H30	10		CMY2	2 (1.64)
26	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O4:H30	1		CMY2	2 (1.64)
27	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O4:H30	10		CMY2	1 (0.82)
28	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O4:H45	1		CMY2	1 (0.82)
29	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O$^-$:H51	3		CMY2	1 (0.82)
30	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H$^+$	19		CMY2	1 (0.82)
31	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O80:H3 or O80:H16	6		CMY2	1 (0.82)
32	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H15	7		CMY2	1 (0.82)
33	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H16	18		CMY2	1 (0.82)
34	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O8:H19	20		CMY2	1 (0.82)
35	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	11		CMY2	7 (5.74)
36	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7		CMY2	47 (38.52)
37	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7		AmpC	1 (0.82)
38	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7			1 (0.82)
39	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7		TEM	1 (0.82)
40	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7		AmpC	1 (0.82)
41	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7	CNF2	CMY2	1 (0.82)
42	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H25	7	STa	CMY2	1 (0.82)
43	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H30	7		CMY2	1 (0.82)
44	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H30	7	CNF2	CMY2	1 (0.82)
45	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H46	7		CMY2	1 (0.82)
46	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O86:H51	15		CMY2	1 (0.82)
47	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O86:H51	4		CMY2	1 (0.82)
48	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O86:H51	4		CMY2	2 (1.64)
49	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H9	14		CMY2	2 (1.64)
50	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O8:H9	7		CMY2	1 (0.82)
51	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H9	14		TEM, AmpC	1 (0.82)
52	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H9	14		CMY2	3 (2.46)
53	Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef	O$^-$:H9	18		CMY2	1 (0.82)
54	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	O$^-$:H9	21		CMY2	1 (0.82)
55	Amp-Spt-Tet-Cef	O$^-$:H9	26		CMY2	1 (0.82)
56	Amp-Chl-Ffc-Tet-Tic-Tim-Cef	O9:H4	23		CMY2	1 (0.82)
57	Amp-Chl-Gen-Ffc-Tet-Tic-Tim-Cef	OR:H51	4		CMY2	1 (0.82)
58	Amp-Spt-Tet-Tic-Tim-Cef	Ox43:H16	18		TEM	1 (0.82)
59	Amp-Spt-Tet-Tic-Tim-Cef	Ox43:H2	18	STa	CMY2	1 (0.82)

ing eight virulence factors. The prevalence of virulence gene products, including STa (3.3%), Stx1 (0.82%), Stx2 (0%), CNF1 (0%), CNF2 (1.63%), K99 (0%), CS31A (0.82%), and F1845 (3.3%) was low. Only 10 of 122 isolates (8%) encoded virulence gene products (Table 3).

PCR detection of beta-lactamase genes. All Cef^r *E. coli* isolates were screened for the cephamycinase bla_{CMY2} gene by a PCR-based assay. A total of 117 isolates (96%) were positive for the gene (Table 5). The six isolates negative for bla_{CMY2} were screened for other beta-lactamase genes, including

TABLE 4. MICs for Cef^r *E. coli* isolates

Antimicrobial	MIC (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)[c]	No. of resistant isolates (%)
Ampicillin	32[a]	512	512	128–>512 (1.7–9.8)	122 (100)
Ceftiofur	8	64	128	16–256 (14–5.7)	122 (100)
Chloramphenicol	32	256	>256	8–>256 (0.8–10.7)	121 (99)
Florfenicol	8	256	>256	8–>256 (0.8–25)	122 (100)
Gentamicin	16	32	64	2–512 (7.4–1.6)	100 (82)
Spectinomycin	128	>1,024	>1,024	4–>1024 (0.8–63)	101 (83)
Tetracycline	16	128	512	2–>512 (0.8–1.6)	121 (99)
Ticarcillin	128	>1,024	>1,024	128–>1,024 (24.6–58)	122 (100)
Ticarcillin/clavulanic acid[b]	128 [2]	256 [2]	512 [2]	64 [2]–1024 [2] (0.8–7.4)	121 (99)

[a] Breakpoint MIC according to NCCLS document M31-A2 (25).
[b] Data for clavulanic acid are shown in brackets.
[c] Values in parentheses are percentages of isolates at minimum and maximum MIC range values.

bla_{CTX-M2}, bla_{FOX}, bla_{SHV}, bla_{TEM}, and *ampC*. The *ampC* primers recognized any plasmid-borne cephamycinase gene other than bla_{CMY2}. Three isolates carried the bla_{TEM} gene, and three isolates were positive for *ampC* (Table 3). One isolate (ceftiofur MIC, 128 μg/ml) carried both bla_{TEM} and *ampC* genes. None of the isolates contained the bla_{CTX-M2}, bla_{FOX}, or bla_{SHV} gene. A single isolate (ceftiofur MIC, 32 μg/ml) was negative for all the beta-lactamase genes included in the PCR assays.

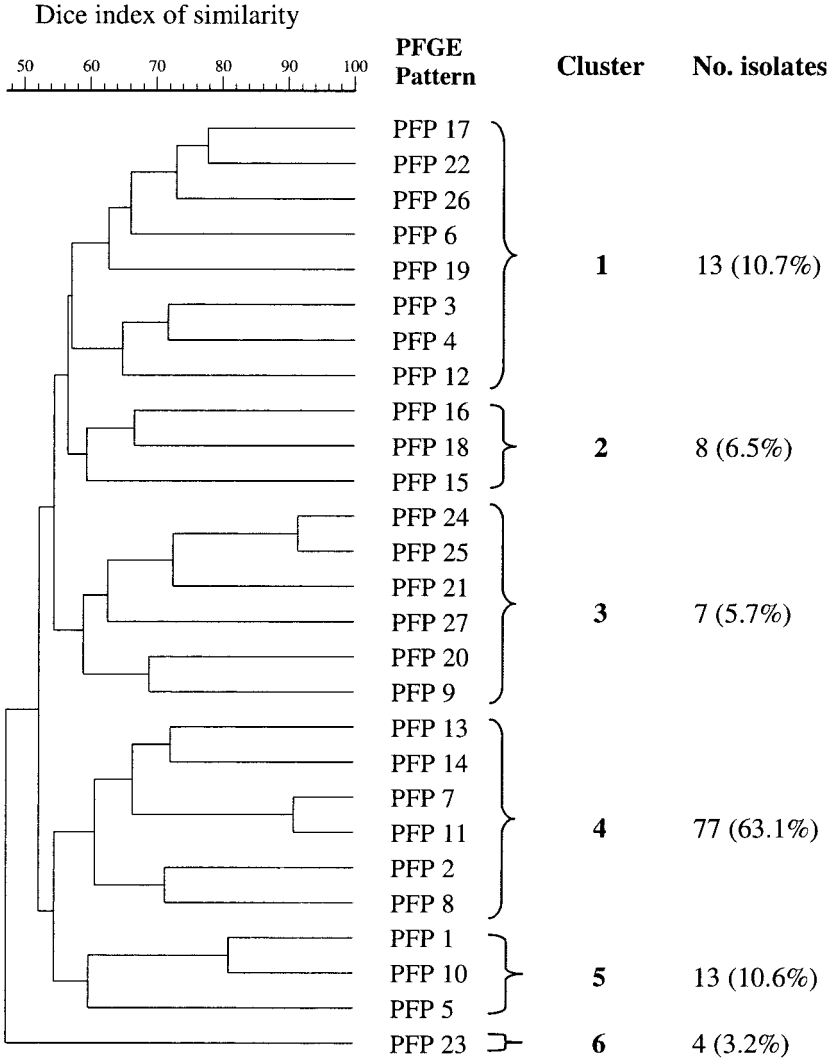

FIG. 1. Dendrogram showing the relationships between PFGE profiles of Cef^r *E. coli* isolates.

TABLE 5. Prevalence of beta-lactamase genes in Cef^r *E. coli* isolates

bla$_{CMY2}$ gene	No. of isolates with indicated ceftiofur MIC (μg/ml)				
	16 (n = 17)	32 (n = 34)	64 (n = 31)	128 (n = 34)	256 (n = 6)
Positive	16	32	30	32	6
Negative	1	1	1	3	0

Clonal type analysis. Based on phenotypic (antibiogram and serotype) and genotypic (PFGE profile, virulence and beta-lactamase gene) characteristics, the 122 isolates were classified into 59 clonal types (Table 3). Ten CTs (18%) were isolated multiple times, while 43 CTs (81%) were represented by one isolate. Isolates from August displayed the greatest diversity (21 CTs), while only 7 CTs were observed during May. Clonal types from older calves (≥6 weeks old) were less diverse (8 CTs) than CTs from 3- and 4-week-old calves (13 CTs). Clonal type 36 (n = 47 isolates) was isolated from 42 calves and found in all age groups. This *E. coli* CT belonged to the O8:H25 serotype, exhibited PFGE profile 7, and had a Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef resistance profile. Clonal type 36 carried the *bla*$_{CMY2}$ cephamycinase gene, as determined by PCR (Table 3). The incidence of CT 36 was analyzed with respect to calf age and sampling month using a chi-square test of independence (Table 6). There was no significance for the incidence CT 36 by calf age ($P < 0.05$); however, there was statistical significance for the incidence of CT 36 by the month of sampling ($P < 0.001$).

TABLE 6. Significance of clonal type 36 isolated from dairy calves

Sample time	CT 36	Other CT	Total CTs
Month[a]			
April	0	16	16
May	14	6	20
June	11	19	30
July	14	10	24
August	8	24	32
Total	47	75	122
Calf age (wk)[b]			
1	5	9	14
2	13	10	23
3	5	16	21
4	8	14	22
5	6	10	16
6	4	8	12
>7	6	8	14
Total	47	75	122

[a] Test of significance for CT 36 and month, χ^2 (P) = 24.885 (0.000).
[b] Test of significance for CT 36 and calf age, χ^2 (P) = 5.410 (0.492).

DISCUSSION

Studies of antimicrobial resistance of intestinal *E. coli* from different animal species show an increase in the prevalence of resistance over time as the result of widespread use of antimicrobial drugs (18, 26, 34). Various levels of ceftiofur resistance have been reported in studies of *E. coli* from calves. White et al. (41) reported a prevalence rate of 69% of ceftiofur-resistant *E. coli* isolates from diarrheic calves, while Bradford et al. (7) reported a prevalence rate of only 13%. Similar low levels of ceftiofur resistance have been reported for *E. coli* and *Salmonella* strains in Europe (6). A recent study of *Salmonella* at a heifer replacement farm in Texas observed that 86% of *Salmonella* spp. were resistant to ceftiofur (5). In our study, 88.5% of calves shed Cef^r GNEB. A higher prevalence rate observed in our study could be attributed to (i) the isolation and detection of Cef^r GNEB technique employed in the study, (ii) isolates that were from healthy calves, compared to the diarrheic or sick calves reported by other researchers (7, 41), or (iii) the poor management of antibiotic use on this particular farm.

The number of gram-negative bacteria, *E. coli* in particular, in calf feces is higher than that in adult cattle feces (18). The number of GNEB decreased as calf age increased, as shown in other studies (17, 18). The decrease in the prevalence of Cef^r GNEB could be due to the overall decrease in total fecal *E. coli*. The decline in the proportion of Cef^r GNEB may be related to changes in competition due to ruminal development and diet as the animals are weaned. Housing and dietary changes, such as weaning, may affect the prevalence of antibiotic-resistant strains by altering the calf's exposure to other animal stock and colonizing strains or changing the *E. coli* composition of the gut microflora (16).

Several classes of antibiotics are approved for the treatment of scours and enteritis in dairy calves in the United States (10). A recent survey of Pennsylvania dairy producers reported tetracycline, neomycin, and spectinomycin as the most commonly used antibiotics in treatment of enteritis in calves (33). Zwald et al. (46) found that 78% of the farms surveyed used antibiotics in the treatment of calf diarrhea, and the use of ceftiofur to treat calf diarrhea was reported by 11% of the farms. Treatment of bovine respiratory disease was the most commonly reported use of ceftiofur in both surveys (33, 46).

In this study, all *E. coli* isolates were resistant to three or more antibiotics. The most common antibiogram pattern observed was Amp-Chl-Gen-Ffc-Spt-Tet-Tic-Tim-Cef. The high level of multidrug resistance in healthy dairy calves has not been reported in previous studies. In a study by Werckenthin et al. (40), *E. coli* isolates from diarrheic calves were screened against a panel of 13 antibiotics, with 63% showing resistance to multiple drugs; 23% were resistant to nine or more drugs. Orden et al. (27) tested *E. coli* isolates from diarrheic calves against a similar panel of antibiotics and observed that 76.9% showed resistance to at least two antibiotics but that only 32% were resistant to eight or more antibiotics. We found the highest prevalence of multidrug resistance in 2-week-old calves. Remarkably, even day-old calves shed *E. coli* isolates resistant to 9 of the 10 antibiotics tested; other researchers have made similar observations (27, 40).

We observed that 93% of the Cef^r *E. coli* isolates were resistant to both florfenicol and chloramphenicol. Florfenicol and chloramphenicol belong to the thiophenicol group of antibiotics. Although the use of chloramphenicol in food animals has been banned in the United States since the 1980s, the resistance observed in this study could be due to genes encoding florfenicol resistance. Florfenicol was approved by the Food and Drug Administration (FDA) in 1996 for the treat-

ment of bovine respiratory disease (41). A similar level of cross-resistance has been reported by White et al. (41). They showed that 85% of *E. coli* isolates from diarrheic dairy calves were resistant to both chloramphenicol and florfenicol. Similar cross-resistance has also been reported for *Salmonella* spp. (2, 4). All *E. coli* strains were sensitive to the fluoroquinolone antibiotic Eno. Fluoroquinolone drugs are highly effective against *E. coli* infections in animals; however, emerging resistance to fluoroquinolones has been reported in Europe (8, 40). The findings of this study suggest that dairy-origin GNEB in Pennsylvania are not likely to be a reservoir of fluoroquinolone resistance genes.

The Cef^r *E. coli* isolates exhibited elevated MICs for several unrelated classes of antibiotics. The MIC_{90} for ceftiofur was threefold higher than the NCCLS breakpoint value. A study by Sato et al. (32) reported ceftiofur MICs ranging from 0.5 to 16 μg/ml for *E. coli* samples from healthy calves; while Bradford et al. (7) reported ceftiofur MICs ranging from 0.25 to >128 μg/ml for *E. coli* samples from diarrheic calves. Elevated MICs for ampicillin (>128 μg/ml), ticarcillin (32 to >128 μg/ml), and ticarcillin-clavulanate (16 to >128 μg/ml) were also observed (7). Orden et al. (27) reported that *E. coli* isolates from diarrheic calves had elevated MIC_{90} values for ampicillin (>512 μg/ml), spectinomycin (>512 μg/ml), gentamicin (32 μg/ml), chloramphenicol (512 μg/ml), and tetracycline (256 μg/ml). Similar MIC_{90} values were observed in this study (Table 4).

Serotyping continues to be a fundamental method used to classify *E. coli* isolates and is considered the "gold standard." In a study on the ecology of *E. coli* in cattle, Hinton et al. (15) isolated 55 different O serogroups from 16 calves, whereas Glantz (13) reported 88 different O:H serotypes associated with *E. coli* isolates from calves. We found that 57.3% of the Cef^r *E. coli* isolates belonged to the O8 serogroup and 49.2% belonged to serotype O8:H25. Interestingly, Glantz (13) also observed *E. coli* belonging to the O8 serogroup as the predominant O type isolated from young dairy calves. The O8 serogroup has been associated with diarrhea and extraintestinal infections in calves (23). In our study, other serogroups, including O4, O86, O106, and O$^-$ or nontypeable strains, were identified. The O4 and O86 serogroups have been reported to be associated with calf scours (26, 39), and nontypeable *E. coli* strains have been isolated from bacteremic calves (12).

PFGE typing is often used to determine sources of foodborne outbreaks by comparing human isolates with those from food and animals. In this study, PFGE analysis of 122 Cef^r *E. coli* isolates resulted in 27 PFPs, with 77 of 122 isolates (63.1%) belonging to a single cluster. PFGE and serotype analyses showed that cluster 4 on the phylogenetic tree contained nearly 60 isolates that had the same PFP and serotype. PFGE profiles correlated well with O serogroup and antibiotic resistance. A similar correlation between antibiotic resistance and PFGE profile has been seen in multidrug-resistant *Salmonella enterica* serotype Newport (4, 31).

Most studies of antibiotic resistance in animal agriculture have been directed toward pathogenic bacteria (6, 7, 11, 29, 42). The findings of this study provide a unique perspective on the role of commensal *E. coli* as a potential reservoir of resistance genes for multiple antibiotics. Monitoring resistance in commensal bacteria, such as *E. coli*, is important, as they can gain access to the food chain. Zhao et al. (45) reported the presence of extended-spectrum cephalosporin-resistant *E. coli* and *Salmonella* spp. in retail ground meat, signifying the public health importance of this issue. Nonpathogenic multidrug-resistant strains of *E. coli* in the intestinal microflora serve as an important reservoir of mobile resistance genes which can be transferred in the intestines to other bacterial species, including pathogens such as *Salmonella* spp. (1, 5, 16, 17, 37). This can be an important mechanism for acquiring antibiotic resistance in pathogenic bacteria that pose a challenge for effective antibiotic therapy. The findings of our study suggest that commensal *E. coli* can perhaps play a dynamic role in the ecology of multidrug resistance in the dairy environment.

The most common mechanism of cephalosporin resistance is through production of beta-lactamases. Broad-spectrum cephalosporins, like ceftiofur, have increased ring stability against some beta-lactamases but are susceptible to cephamycinases (20). Cephamycinases (bla_{CMY}) share extensive homology to chromosomal *ampC* beta-lactamases. The bla_{CMY2} gene is closely related to the chromosomal *ampC* of *Citrobacter freundii* (44). Ceftiofur resistance in *E. coli* is typically due to the presence of the bla_{CMY2} gene (45). Our findings support this, as the bla_{CMY2} gene was found in 96% of the ceftiofur-resistant *E. coli* isolates in this study. For one Cef^r *E. coli* isolate, none of the acquired beta-lactamase genes assayed in this study were detected. There are two possible mechanisms for ceftiofur resistance in this isolate, (i) presence of a cephamycinase gene not included in our assay (30) or (ii) mutations in the *E. coli* chromosomal *ampC* gene, causing hyperproduction of this enzyme (36).

There is concern that the widespread use of ceftiofur in cattle is selecting for and maintaining ceftriaxone-resistant *Salmonella* spp. in the intestinal flora of cattle (1, 5). Ceftriaxone, an analogue of ceftiofur, is an expanded-spectrum cephalosporin used for the treatment of *Salmonella* infections in children. In the cases of domestically acquired infections, most ceftriaxone-resistant *Salmonella* spp. carry bla_{CMY2} (43). Researchers have shown that transfer of the bla_{CMY2} gene between *E. coli* and *Salmonella* can occur (3, 44).

We used several methods to classify the ceftiofur-resistant *E. coli* isolates in this study. A total of 59 Cef^r *E. coli* clonal types were identified, of which CT 36 was the most prevalent. Clonal type 36 appeared suddenly as the predominant CT, having not been observed in the preceding month, and continued to be the most prevalent CT for several months. The results show clonal expansion of this particular *E. coli* strain among the calf herd. We speculate that the source of this CT was the maternity pen, calf-to-calf contact, contact with farm workers, or a common source, such as contaminated water. Additionally, this farm did not keep records of antibiotic treatment, meaning that the misuse of antibiotics could be contributing to the high level of multidrug resistance seen among these calves.

In conclusion, we found that healthy dairy calves were rapidly colonized by antibiotic-resistant strains of *E. coli* shortly after birth. A recent study of the dynamics of *E. coli* in dairy calves showed that antibiotic-resistant strains had a greater selective advantage in newborn calves than antibiotic-susceptible strains, even in calves not treated or exposed to antibiotics (18). A selective advantage for antibiotic-resistant *E. coli* strains to thrive in the intestinal environment of young calves could explain why multidrug-resistant strains were isolated

from day-old calves in this study. A significant public health concern is that multidrug-resistant commensal *E. coli* strains may constitute a potential reservoir of resistance genes that could be transferred to pathogenic bacteria (9). The findings of our study provide evidence to support earlier studies that suggest the existence of a reservoir of antibiotic resistance genes (7, 16). Infections with multidrug-resistant pathogens limit the options available to treat infectious disease of animals and humans. The high prevalence of multidrug-resistant bacteria observed in this study suggests there is a need for improved education and communication on the issue of antibiotic use between dairy farmers and veterinarians. Young dairy calves are susceptible to many pathogens, and infections with antibiotic-resistant organisms may lead to treatment failure that can result in economic losses for the producer. With the continuing emergence of antibiotic resistance, it is imperative that actions be taken to prolong the effectiveness of existing antibiotics while maintaining levels of food animal production.

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