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Antimicrobial-Resistant Enteric Bacteria from Dairy Cattle▽

Ashish A. Sawant,[1] Narasimha V. Hegde,[1] Beth A. Straley,[1] Sarah C. Donaldson,[1] Brenda C. Love,[1] Stephen J. Knabel,[2] and Bhushan M. Jayarao[1]*

Department of Veterinary and Biomedical Sciences[1] and Department of Food Science,[2] Pennsylvania State University, University Park, Pennsylvania

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A study was conducted to understand the descriptive and molecular epidemiology of antimicrobial-resistant gram-negative enteric bacteria in the feces of healthy lactating dairy cattle. Gram-negative enteric bacteria resistant to ampicillin, florfenicol, spectinomycin, and tetracycline were isolated from the feces of 35, 8, 5, and 42% of 213 lactating cattle on 74, 39, 9, 26, and 82% of 23 farms surveyed, respectively. Antimicrobial-resistant gram-negative bacteria accounted for 5 (florfenicol) to 14% (tetracycline) of total gram-negative enteric microflora. Nine bacterial species were isolated, of which *Escherichia coli* (87%) was the most predominant species. MICs showing reduced susceptibility to ampicillin, ceftiofur, chloramphenicol, florfenicol, spectinomycin, streptomycin, and tetracycline were observed in *E. coli* isolates. Isolates exhibited resistance to ampicillin (48%), ceftiofur (11%), chloramphenicol (20%), florfenicol (78%), spectinomycin (18%), and tetracycline (93%). Multidrug resistance (≥3 to 6 antimicrobials) was seen in 40% of *E. coli* isolates from healthy lactating cattle. Of 113 tetracycline-resistant *E. coli* isolates, *tet*(B) was the predominant resistance determinant and was detected in 93% of isolates, while the remaining 7% isolates carried the *tet*(A) determinant. DNA-DNA hybridization assays revealed that *tet* determinants were located on the chromosome. Pulsed-field gel electrophoresis revealed that tetracycline-resistant *E. coli* isolates ($n = 99$ isolates) belonged to 60 subtypes, which is suggestive of a highly diverse population of tetracycline-resistant organisms. On most occasions, *E. coli* subtypes, although shared between cows within the herd, were confined mostly to a dairy herd. The findings of this study suggest that commensal enteric *E. coli* from healthy lactating cattle can be an important reservoir for tetracycline and perhaps other antimicrobial resistance determinants.

Widespread reliance on antimicrobials in food animal production has resulted in a considerable rise of antimicrobial-resistant strains of bacteria, complicating the treatment of infectious diseases in livestock, companion animals, and humans. This has led to important changes in the perceptions and priorities of federal agencies with regard to antimicrobial usage, in particular the use of antimicrobials as growth promoters and prophylactic agents (2, 37, 45).

Sawant et al. (47) conducted a study on antimicrobial usage in Pennsylvania dairy herds. They observed that antimicrobials were used frequently for treating enteritis (36%) and pneumonia (25%) in calves and foot infections in adult cattle (16%). Twenty-four antimicrobials, including beta-lactams, spectinomycin, florfenicol, and tetracyclines, were used on these farms. Beta-lactam antimicrobials were used mostly for dry cow therapy, for clinical mastitis, and, on some farms, for pneumonia and metritis. On 18% of the dairy herds surveyed, ceftiofur was used in an extra-label manner to treat mastitis in lactating cattle. On 70% of farms, calves were fed medicated milk replacers containing oxytetracycline and neomycin. The results of the study by Sawant et al. suggested that antimicrobials were used extensively on dairy herds for both therapeutic and prophylactic purposes. Beta-lactams and oxytetracyclines were the most widely used antimicrobials.

The selective pressure from the use of antimicrobial agents at subtherapeutic levels in dairy cattle could result in the selection of those strains that contain genes for antimicrobial resistance (37). Most of the antimicrobial susceptibility studies reported in the literature have examined pathogenic bacteria, such as *Salmonella* and enterotoxigenic *Escherichia coli*, or bacteria isolated from clinical cases (42, 54). Diagnostic laboratories focus primarily on the detection and characterization of specific pathogenic bacteria. The use of data compiled from routine diagnostic workups may prove to be insufficient to measure the relevance of antimicrobial-resistant normal gut flora of animals or humans (37). Recent studies have shown that commensal bacteria of humans and animals could serve as good indicators of antimicrobial selective pressure and reveal the potential for antimicrobial resistance emerging in enteric pathogens (29). The French Institute for Public Health Surveillance has recommended that studying antimicrobial resistance in commensal bacteria from healthy animals would be extremely valuable, as these organisms could serve as a reservoir for genes that encode antimicrobial resistance and, given the right conditions, could transfer these resistance genes to pathogenic bacteria (15).

The tetracyclines continue to be one of the most widely used antibiotics in human medicine and animal agriculture, as they are relatively inexpensive, can be administered orally, and have relatively few side effects (10). The growth-promoting properties of oxytetracyclines were first reported by Stockstad et al. (53), when young chicks fed with chlortetracycline showed improvement in growth rate. Following this report, chlortetracycline and oxytetracycline were extensively used as animal growth promoters in swine and cattle (16).

* Corresponding author. Mailing address: Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, PA 16802. Phone: (814) 863-5939. Fax: (814) 863-6140. E-mail: bmj3@psu.edu.

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Resistance to tetracyclines was first detected in the 1950s and became more apparent by the 1970s when it was widely reported among *Enterobacteriaceae*, staphylococci, streptococci, and *Bacteroides* spp. (30). Tetracycline resistance determinants are now widely spread among bacterial species and have been identified in as many as 39 gram-negative and 22 gram-positive bacteria (10). In most gram-negative species, tetracycline resistance is due to the acquisition of an operon which consists of an efflux gene *tet*(A) and a repressor gene *tet*(R) that are divergently transcribed from overlapping operator regions. Nine types of efflux genes consisting of the above operon have been described so far for gram-negative bacteria [*tet*(A) to *tet*(E) and *tet*(G) to *tet*(J)] (48).

This study was conducted to determine (i) the prevalence and distribution of antimicrobial-resistant gram-negative enteric bacteria (GN-EB) from healthy lactating cattle, (ii) the MICs of commonly used antimicrobials against *E. coli* isolated from the feces of lactating dairy cattle, and (iii) genotypic characteristics of tetracycline-resistant *E. coli*. It is anticipated that the findings of this study will provide a comprehensive understanding of antimicrobial resistance in commensal bacteria isolated from the feces of healthy lactating cattle.

MATERIALS AND METHODS

Dairy cattle. A total of 23 herds from central and south-central Pennsylvania (four counties) were surveyed for antimicrobial-resistant GN-EB in the feces of healthy lactating cattle. These herds had participated earlier in a study on food-borne pathogens in bulk tank milk (20) and antimicrobial usage (47). Dairy producers kept records on the type of antimicrobials that were used and the purpose for which they were administered. However, information on the dose, frequency of use, animals that received antimicrobials, and whether antimicrobial treatment was completed was not available for all of the farms. Therefore, no attempt was made to correlate the use of an antimicrobial with the occurrence of antimicrobial-resistant bacteria. All dairy herds used ampicillin, florfenicol, spectinomycin, and tetracycline. Fecal samples from 213 lactating cattle (n = 23 herds) were screened for antimicrobial resistance. From each herd, the number of lactating cattle that was sampled ranged from 4 to 12 animals (approximately 10% of the lactating cattle present in the milking herd). Fecal samples were randomly collected from lactating cattle that exited the milking parlor or those restrained in a tie stall for bacteriological analysis. Feces were collected from cows per rectal using a sterile disposable rectal sleeve. About 10 g of feces was transferred to a sterile 50-ml screw-cap centrifuge tube. The tube was transported to the laboratory on ice and processed the same day.

Isolation and enumeration of GN-EB and antimicrobial-resistant GN-EB. The fecal samples were thoroughly mixed using a sterile spatula, and 1 g of feces was transferred to a 15-ml centrifuge tube containing 9 ml of sterile normal saline solution. The contents were mixed thoroughly and serially diluted 10-fold. MacConkey agar (MAC) was used for selective growth of GN-EB. From the 10^{-3} and 10^{-4} dilutions, 0.1 ml was plated on a MAC control plate without antimicrobials, while from the 10^{-1} and 10^{-2} dilutions, 0.1 ml of the sample was plated on a MAC plate containing one of the following antimicrobials: ampicillin (64 μg/ml), tetracycline (32 μg/ml), spectinomycin (256 μg/ml) (ICN Biomedicals, Aurora, OH), enrofloxacin (8 μg/ml) (Sigma Aldrich, St. Louis, MO), or florfenicol (16 μg/ml) (Sigma Aldrich, St. Louis, MO). The concentration of antimicrobials used in MAC media was one "twofold-higher concentration" than the MIC breakpoint values suggested by the CLSI (formerly NCCLS) (39) for bacteria isolated from animals. The inoculated plates were incubated at 37°C for 24 h. The numbers of colonies on MAC with antimicrobials and without antimicrobials were counted and expressed as antimicrobial-resistant GN-EB CFU/g of feces and total GN-EB CFU/g of feces, respectively.

Species identification of antimicrobial-resistant GN-EB. A total of 258 isolates (n = 23 farms; 213 lactating cattle) from MAC with antimicrobials were identified to the species level. Based on colony morphology and lactose fermentation, two to three colonies were selected from each plate for species identification. Colonies were tested by Gram reaction, oxidase testing, and indole–methyl red–Voges-Proskauer–citrate battery testing as described by Harley and Prescott (18). The isolates were then identified to the species level using an API 20E/NE identification kit (bioMérieux, Hazelwood, MO) as described by the manufacturer.

Antimicrobial susceptibility assays. All *E. coli* isolates (n = 223) out of 258 GN-EB were examined for susceptibility to antimicrobials by broth microdilutions as described by the CLSI (39). The isolates were screened, and the results were interpreted according to criteria established by the CLSI (39) for bacteria isolated from animals. MICs of *E. coli* for ampicillin, chloramphenicol, spectinomycin, streptomycin, tetracycline (MP Biomedicals, Aurora, OH), ceftiofur, and florfenicol (Sigma Aldrich, St. Louis, MO) were determined as described by the CLSI (39). The MIC for each isolate was read as the lowest dilution demonstrating no visible growth. Based on CLSI recommendations (39), *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 were used as quality control strains for MIC assays. The MIC_{50} and MIC_{90} values were calculated and expressed as the percentage of isolates at each of the respective MICs.

Tetracycline resistance determinants. Tetracycline-resistant enteric *E. coli* isolates (n = 113) from lactating dairy cattle were screened for tetracycline resistance genes *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) as described by Ng et al. (40). The PCR assays were optimized (template and primer concentration) for PuReTaq Ready-To-Go PCR beads (GE Healthcare, Piscataway, NJ).

Pulsed-field gel electrophoresis. Tetracycline-resistant *E. coli* isolates (n = 113 isolates from 18 herds) were subtyped using the pulsed-field gel electrophoresis (PFGE) protocol described by Hegde et al. (19). The PFGE subtypes for 14 isolates could not be determined due to lack of clear discernible DNA banding patterns. The 14 isolates were all subjected to PFGE analysis on three separate occasions; all of the analyses failed to result in clear DNA banding patterns. The PFGE patterns were first compared visually and analyzed using Gel Doc 2000 Molecular Analyst Fingerprinting Plus, version 6.1, software (Bio-Rad, Hercules, CA). *Salmonella enterica* serovar Newport was used as a reference strain for PFGE analysis.

Southern blot hybridization assays. Small- and large-sized plasmids were obtained using an alkaline lysis protocol with sodium dodecyl sulfate as described by Sambrook and Russell (46). Small-sized plasmids (≤12 kb) were electrophoresed on a 0.8% agarose gel, while plasmids of large sizes that may be difficult to obtain on regular gel electrophoresis were resolved by PFGE using a 1% agarose gel. Genomic DNA-PFGE was performed using the 1-day PFGE protocol developed by Hegde et al. (19). The bacterial plugs were digested with XbaI and run on the CHEF Mapper system (Bio-Rad). The running conditions for large plasmids and genomic DNA were as follows: initial switch time, 2.16 s; final switch time, 35.07 s; run time, 14 h; angle, 120°, gradient, 6.0 V/cm; temperature, 14°C (ramping factor was kept linear). Gels were blotted with a vacuum blotter according to the manufacturer's instructions (Bio-Rad Laboratories, CA). A positively charged Hybond N^+ nylon membrane (GE Healthcare) was used for blotting. Small plasmids were blotted for 90 min, while large plasmids and genomic DNA were first depurinated (0.25 N HCl for 15 min) and then blotted for 180 min.

PCR-amplified products *tet*(A) and *tet*(B) were purified and used as probes for the Southern blotting assay. A digoxigenin (DIG) DNA-labeling and detection kit (Roche Molecular Biochemicals, Mannheim, Germany) was used to randomly label DNA probes. Briefly, *tet*(A) and *tet*(B) amplified products were denatured by heating in a boiling water bath for 10 min and immediately chilled on ice. Random hexanucleotides (10×, 2 μl) were added to hybridize to the denatured template. Klenow enzyme (1 μl) was added to create a complementary strand to the template, using the hybridized hexonucleotides as primers and a mix of deoxynucleoside triphosphates (2 μl) containing DIG-labeled dUTP for elongation. The reaction mixture was incubated overnight at 37°C and kept at −20°C until further use. The DIG-labeled probes were used to detect blotted nucleic acids by standard Southern blot methods described by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). The hybridized probes were visually detected with anti-digoxigenin-alkaline phosphatase Fab fragments after the addition of the colorimetric substrate Nitro Blue Tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate).

RESULTS

Prevalence of antimicrobial-resistant GN-EB. Ampicillin-resistant GN-EB were isolated from 72 of 211 (34%) of cows on 17 (74%) dairy herds. Tetracycline-resistant GN-EB were isolated from 89 of 212 (42%) cows on 19 (82%) dairy herds. Florfenicol- and spectinomycin-resistant GN-EB were also detected in the feces of healthy cattle; however, their prevalence

TABLE 1. Prevalence and distribution of antimicrobial-resistant and total GN-EB in feces of healthy lactating dairy cattle from 23 farms

Antimicrobial	Total no. of cows screened	Distribution of antimicrobial-resistant GN-EB		Prevalence of antimicrobial-resistant GN-EB		Prevalence of total GN-EB		% of resistant bacteria (% of total GN-EB)	
		No. (%) of farms	No. (%) of cattle	Range (CFU/g of feces)	Mean (CFU/g of feces)	Range (CFU/g of feces)	Mean (CFU/g of feces)	Range	Mean
Ampicillin	211	17 (74)	72 (34)	4×10^1–1.9×10^7	3.1×10^5	4.9×10^3–5.5×10^7	3.8×10^6	0.005–96	9
Enrofloxacin	213	0 (0.0)	0 (0.0)			40–8.5×10^7	3.3×10^6		
Florfenicol	213	9 (39)	18 (8)	1×10^2–1.2×10^4	2.9×10^2	1×10^4–1.1×10^7	1.2×10^6	0.004–63	5
Spectinomycin	213	6 (26)	10 (5)	1×10^2–3.2×10^5	6.2×10^4	3×10^4–1.1×10^7	1.9×10^6	0.004–89	10
Tetracycline	212	19 (82)	89 (42)	4×10^1–6.7×10^6	3.9×10^5	6×10^2–8.5×10^7	6.9×10^6	0.01–100	14

was lower than that observed for tetracycline and ampicillin. Enrofloxacin-resistant GN-EB were not detected (Table 1). Due to laboratory errors related to the enumeration of bacterial count or the contamination of selective media, ampicillin and tetracycline counts could not be determined for two and one fecal sample, respectively. The number of total GN-EB (on MacConkey agar without antimicrobial agent) ranged from 1.2×10^6 to 6.9×10^6 CFU/g of feces. The mean CFU/g of ampicillin-resistant GN-EB (3.1×10^5 CFU/g) and tetracycline-resistant GN-EB (3.9×10^5 CFU/g) were 1 log lower that of the total GN-EB. On average, the ampicillin- and tetracycline-resistant bacteria accounted for 9 and 14% of total GN-EB, respectively. Florfenicol- and spectinomycin-resistant GN-EB were 3 and 2 log lower than total GN-EB, respectively (Table 1).

Antimicrobial-resistant GN-EB species. A total of 258 antimicrobial-resistant GN-EB isolates from 213 cows on 23 dairy herds were identified to species (Table 2). The antimicrobial-resistant GN-EB ($n = 258$) belonged to 13 gram-negative bacterial species, including *Citrobacter koseri*, *Enterobacter aerogenes*, *Escherichia coli*, *Morganella morganii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Kluyvera* spp., *Providencia alcaligenes*, *Providencia stuartii*, *Pasteurella* spp., and *Pseudomonas* spp. *Escherichia coli* ($n = 223$) was isolated from four MAC plates, each plate supplemented with ampicillin, tetracycline, florfenicol, and spectinomycin. A total of eight and five bacterial species were isolated from MAC supplemented with ampicillin and spectinomycin, respectively (Table 2). All of the isolates from MAC supplemented with tetracycline (32 μg/ml) were identified as *E. coli*. Two isolates from MAC supplemented with florfenicol (16 μg/ml) were identified as *Pasteurella* spp.

TABLE 2. Antimicrobial-resistant GN-EB species isolated from lactating cattle

Species	No. of bacteria isolated on MacConkey agar supplemented with:			
	Ampicillin (64 μg/ml)	Florfenicol (16 μg/ml)	Spectinomycin (256 μg/ml)	Tetracycline (32 μg/ml)
Citrobacter koseri	14			
Enterobacter aerogenes	4			
Escherichia coli	75	22	13	113
Klebsiella oxytoca	3			
Klebsiella pneumoniae			1	
Kluyvera spp.			1	
Morganella morganii	1			
Pasteurella spp.		2		
Providencia alcaligenes	1			
Providencia stuartii			1	
Pseudomonas aeruginosa	1			
Pseudomonas fluorescens	5			
Pseudomonas spp.			1	
All isolates	104	24	17	113

MICs for *E. coli* isolates. Interpretation of the MICs based on CLSI criteria (39) for *E. coli* isolates would result in 48, 11, 78, 20, 18, and 93% resistance to ampicillin, ceftiofur, florfenicol, chloramphenicol, spectinomycin, and tetracycline, respectively (Fig. 1 and Table 3). The MICs for *E. coli* showed a distinct bimodal distribution for ampicillin (MIC_{50}, 4 μg/ml; MIC_{90}, 1,024 μg/ml). Nearly 85% isolates showed an MIC of ≤0.25 μg/ml for ceftiofur, while 11% of the isolates exhibited MICs above the CLSI's (39) recommended resistance breakpoint for ceftiofur (Fig. 1 and Table 3). The MIC_{50} for both florfenicol and chloramphenicol was 8 μg/ml. The MIC_{90} values for florfenicol and chloramphenicol were 256 and 128 μg/ml, respectively (Table 3). Based on the CLSI's (39) recommended breakpoint values, 78 and 20% of the isolates were found to be resistant to florfenicol and chloramphenicol, respectively. The most frequently observed MICs for spectinomycin were 8 μg/ml (28.7%) and 16 μg/ml (43%). No clear trend was observed for susceptibility towards streptomycin. Ninety-three percent of isolates exhibited elevated MICs (≥16 μg/ml) for tetracycline. Both the MIC_{50} and MIC_{90} values for tetracycline were 256 μg/ml as 90% of the isolates were inhibited (Table 3).

Resistance patterns of *E. coli* isolates. The 223 *E. coli* isolates belonged to 21 antimicrobial resistance profiles (Table 4). Resistance to only tetracycline was observed in 8.97% of isolates, whereas only four isolates were ampicillin resistant. Florfenicol and tetracycline (35.87%) resistance was the most common pattern of resistance. Multidrug resistance (≥3 to 6 antimicrobials) was observed in 90 (40.4%) of *E. coli* isolates. The major multidrug resistance profile was ampicillin-florfenicol-tetracycline, which was observed in 13.9% of isolates. A significant number of isolates (8.07%) exhibited resistance to all of the six antimicrobials used for screening (Table 4).

Tetracycline-resistant *E. coli* PFGE subtypes. Of the 113 tetracycline-resistant *E. coli* isolates, the PFGE patterns of 99 isolates are presented in these data. Repeated attempts failed to obtain discrete PFGE patterns from 14 isolates. The tetracycline-resistant *E. coli* isolates ($n = 99$) belonged to 60 PFGE subtypes. The *E. coli* subtypes were frequently detected among

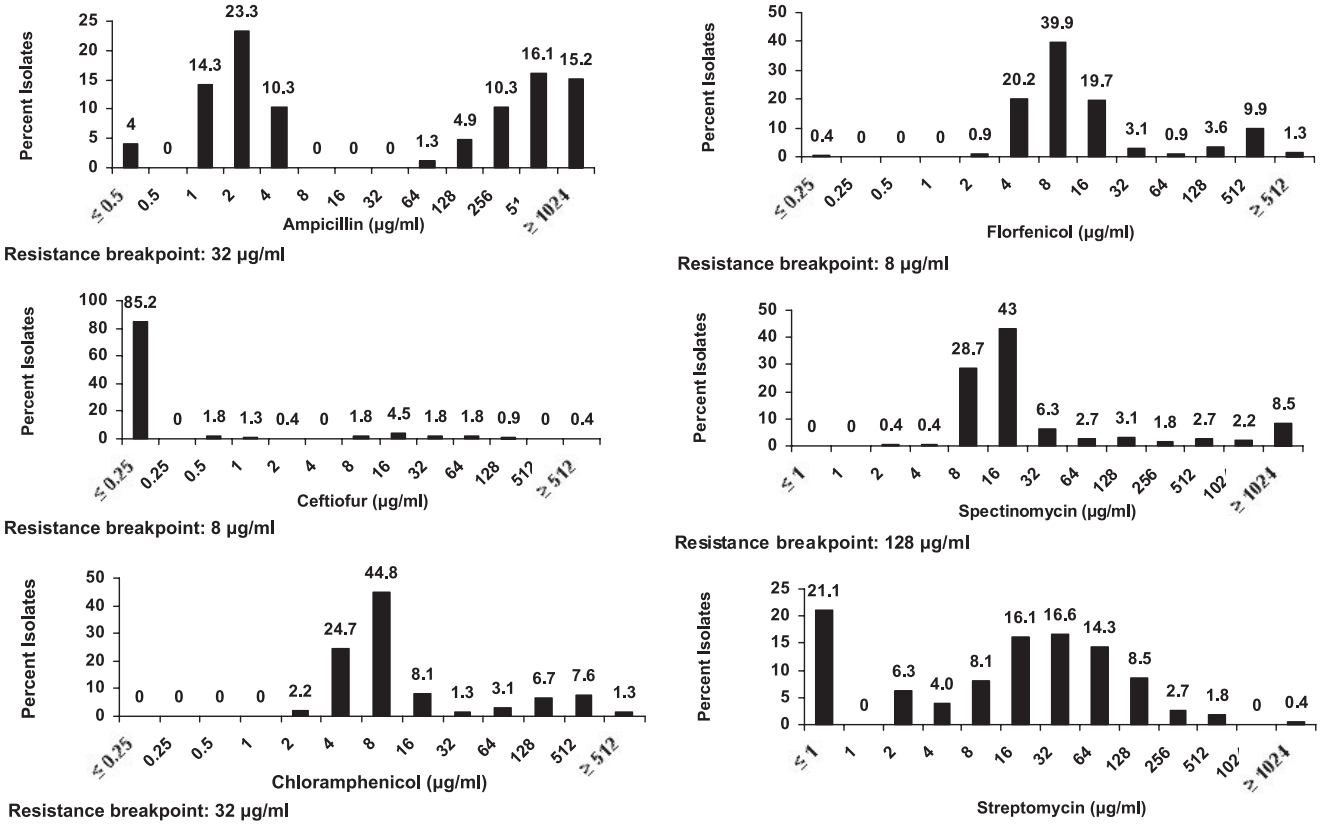

FIG. 1. MICs of 223 *E. coli* isolates for seven antimicrobials. The resistance breakpoints are based on CLSI interpretative criteria (39).

cows within a farm but were rarely observed on more than one farm. The number of tetracycline-resistant *E. coli* PFGE subtypes within a dairy herd ranged from one to eight subtypes. Four subtypes were observed on at least one farm.

Characterization of tetracycline resistance determinants. A total of 113 tetracycline-resistant *E. coli* isolates were analyzed for *tet* genes by PCR analysis. It was observed that 105 (93%) and 8 (7%) isolates encoded *tet*(B) and *tet*(A), respectively. Southern blot hybridizations of small- and large-sized plasmids and genomic DNA from 30 randomly selected isolates with *tet*(B) and all 8 isolates with *tet*(A) were probed with their respective gene probes. No hybridization was seen with either plasmid size. Hybridization of *tet* probes with genomic DNA was detected (Fig. 2). Only one fragment per genomic DNA PFGE lane exhibited a color reaction, indicating that a single copy of *tet*(B) was present in the genome (Fig. 2). The same was observed with *tet*(A) probe (data not shown). The result showed that the efflux genes were located on the genomic DNA.

TABLE 3. Summary of MICs for 223 *E. coli* isolates to antimicrobials

Antimicrobial	MIC (μg/ml)				% Resistant[a]
	Minimum	50%	90%	Maximum	
Ampicillin	≤0.5	4	≥1,024	≥1,024	48
Ceftiofur	≤0.25	≤0.25	8	≥512	11
Chloramphenicol	2	8	128	≥512	20
Florfenicol	≤0.25	8	256	≥512	78
Spectinomycin	2	16	1,024	≥2,048	18
Streptomycin	≤1	16	128	≥2,048	
Tetracycline	≤0.5	256	256	≥1,024	93

[a] Based on CLSI interpretive criteria established for bacteria isolated from animals (39).

DISCUSSION

The study of the prevalence of antimicrobial resistance in commensal microflora can be very useful in monitoring and understanding the process of antimicrobial-mediated selection in individual hosts as well as in the general population (29). MacConkey media supplemented with antimicrobial agents can be effectively used for the isolation of antibiotic-resistant coliforms (25). The isolation of the *flo* gene in gram-negative bacteria with an MIC of ≥16 μg/ml for florfenicol has been more successful than in bacteria with an MIC of 8 μg/ml (breakpoint value recommended by the CLSI [39] for florfenicol) (54). We believed that by using concentrations of antimicrobials higher than those recommended by the CLSI, antibi-

TABLE 4. Resistance patterns of *E. coli* isolates (n = 223) on the basis of MIC breakpoints[a]

Resistance profile	No. of isolates with profile	% of isolates with profile
TET	20	8.97
AMP	4	1.79
FLO-TET	80	35.87
CHL-FLO	3	1.35
AMP-TET	17	7.62
AMP-SPT	1	0.45
AMP-FLO	8	3.59
AMP-FLO-TET	31	13.90
FLO-SPT-TET	1	0.45
CHL-FLO-TET	9	4.04
AMP-SPT-TET	4	1.79
AMP-CHL-TET	2	0.90
CHL-FLO-SPT-TET	3	1.35
AMP-SPT-TET-XNL	1	0.45
AMP-FLO-TET-XNL	1	0.45
AMP-FLO-SPT-TET	9	4.04
AMP-CHL-FLO-TET	3	1.35
AMP-CHL-FLO-TET-XNL	4	1.79
AMP-CHL-FLO-SPT-TET	3	1.35
AMP-FLO-SPT-TET-XNL	1	0.45
AMP-CHL-FLO-SPT-TET-XNL	18	8.07

[a] AMP, ampicillin; CHL, chloramphenicol; FLO, florfenicol; SPT, spectinomycin; TET, tetracycline; XNL, ceftiofur.

otic-resistant enteric bacteria could be effectively isolated with fewer false-positive results.

The high prevalence of tetracycline-resistant (82% of farms, 42% of cows, and 14% of total GN-EB) and ampicillin-resistant (74% of farms, 35% of cows, and 9% of total GN-EB) GN-EB observed in our study suggests that lactating cattle can be a significant reservoir of tetracycline- and ampicillin-resistant GN-EB. Although the prevalences of florfenicol-resistant (39% of farms, 8% of cows, and 5% of total GN-EB) and spectinomycin-resistant (26% of farms, 5% of cows, and 10% of total GN-EB) GN-EB were lower than those of ampicillin- and tetracycline-resistant GN-EB, their role as reservoirs of antimicrobial resistance determinants should not be overlooked.

In an earlier study, Sawant et al. (47) reported that beta-lactams and tetracycline were the most widely used antimicrobials on these dairy herds. They concluded that the absence of antimicrobial treatment records, the lack of written plans for treating sick animals, the failure to consult a veterinarian for treating sick animals, and the failure to complete an antimicrobial treatment course are factors that could lead to the inappropriate use of antimicrobials and the emergence of antimicrobial-resistant bacteria. The variation in the number of farms and cows that shed antimicrobial-resistant GN-EB could be influenced by conditions such as (i) transition of animals from one environment to another, (ii) change in nutrition, (iii) severe weather conditions, and (iv) interaction with other animals in the herd (11, 23, 33, 38).

Lau and Ingham (26) showed that enteric *E. coli* in soil mixed with bovine manure could survive for at least 19 weeks at 9 to 21°C. Mobile genetic elements, such as plasmids, transposons, and integrons, have the ability to maintain and spread antimicrobial-resistant determinants (35). Aminov et al. (1) reported that antimicrobial-resistant GN-EB could serve as an antimicrobial resistance gene pool and facilitate the exchange of antimicrobial genetic determinants with other species in the environment.

Escherichia coli is the predominant bacterial species in the feces of dairy cattle (41). In our study, *E. coli* (86.4%) was the predominant species. Antimicrobial-resistant *E. coli*, *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* spp. are considered opportunistic pathogens, which can cause mastitis, enteritis, pneumonia, and urinary tract infections in dairy cattle (3, 12, 56). Recent studies have shown that *Salmonella* and *E. coli* isolates from humans and animals had the same antimicrobial resistance determinants (13, 31).

Interpretive criteria for *E. coli* isolated from cattle have not been established. In addition, human breakpoints have not been validated for the interpretation of antimicrobial resistance of veterinary isolates (44). The CLSI (39) and Schroeder et al. (49) advise caution in extrapolating the predicted response of a specific animal species to a particular antimicrobial drug, as it may lead to incorrect prediction of the clinical

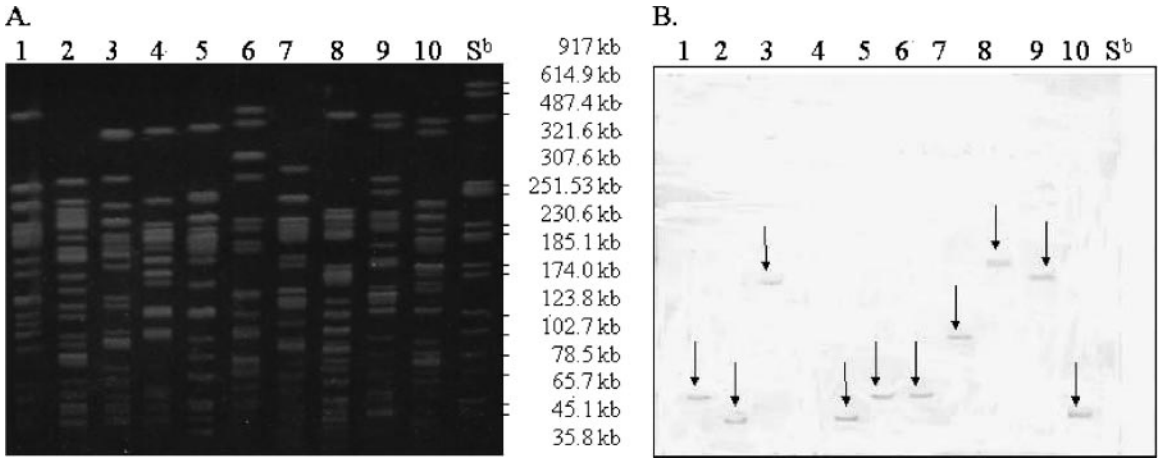

FIG. 2. Southern hybridization of genomic DNA with *tet*(B) probe. (A) Lanes 1 to 10 represent PFGE genomic DNA fragments. (B) Blot of PFGE genomic DNA probed with *tet*(B) probe. The *Salmonella* serovar Newport (S^b) PFGE reference strain (XbaI-digested genomic DNA fragments) is tetracycline sensitive and served as a negative control.

outcome. The caution for extension of interpretation of our data is less likely to be an issue, as the focus of this study was on commensal bacteria from healthy lactating cattle. Nearly 50% of *E. coli* isolates exhibited elevated MICs of ampicillin above the CLSI's (39) recommended resistant breakpoint. The MIC_{90} for ampicillin was as high as ≥1,024 μg/ml. Orden et al. (42) reported a similar observation with *E. coli* isolates from calves with diarrhea. *Escherichia coli* isolates with elevated MICs of ampicillin have been isolated from the feces of healthy lactating cattle and other food-producing animals (22, 44).

The MICs observed for florfenicol- and chloramphenicol-resistant *E. coli* in our study followed a bimodal distribution. Singer et al. (51) reported similar MIC patterns for both antimicrobials in fecal *E. coli* isolates from dairy cattle. They also observed that *E. coli* isolates with an MIC of ≥32 μg/ml harbored the *flo* gene responsible for florfenicol resistance and that isolates without the *flo* gene had an MIC of ≤8 μg/ml. The findings of this study also concur with the observations made by White et al. (54). They reported that the appearance of *E. coli* isolates with MICs of ≥128 μg/ml was a recent phenomenon. They also observed that *E. coli* isolates with elevated MICs for florfenicol (≥16 μg/ml) also exhibited high MICs for chloramphenicol (54).

Of the 43 isolates that showed an MIC of ≥32 μg/ml for florfenicol, an overwhelming majority ($n = 37$; 86%) exhibited MICs at or beyond the resistance breakpoint (≥32 μg/ml) defined by the CLSI (39) for chloramphenicol. Though chloramphenicol has been banned from use in food animals in the United States since the 1980s (14), florfenicol, a structural analog of chloramphenicol, approved by the Food and Drug Administration in 1996, is used for treating bovine respiratory pathogens. Resistance to florfenicol in *E. coli* attributed to the presence of *flo* is also capable of conferring cross-resistance to chloramphenicol (54).

In this study, *E. coli* isolates exhibited very high MICs (MIC_{90}, 1,024 μg/ml) for spectinomycin. Orden et al. (42) also observed that *E. coli* isolates from dairy calves with diarrhea had very high MICs for spectinomycin. Kijima-Tanaka et al. (24) screened *E. coli* isolates from Japanese cattle for resistance to various antimicrobials, including streptomycin.

Researchers categorized 20.8% (>50 μg/ml) of isolates as resistant. At this breakpoint, as well as the resistance breakpoint for streptomycin (64 μg/ml) recommended by the U.S. Food and Drug Administration, the U.S. Department of Agriculture, and the Centers for Disease Control and Prevention standard, 27.7% of *E. coli* isolates from our study were classified as resistant to streptomycin. Bywater et al. (8) observed a similar MIC_{90} value (128 μg/ml) for *E. coli* isolated from cattle in Denmark and Italy. *Escherichia coli* isolates collected by this group from France, Germany, and United Kingdom exhibited a much lower MIC_{90} value (8 μg/ml) for the same antimicrobials (8).

Blake et al. (5) observed that *E. coli* isolates that carried the *tet* genes exhibited high MICs. They observed that *E. coli* isolates from animal feces devoid of tetracycline resistance genes exhibited an average MIC of 1.25 μg/ml, while *E. coli* isolates with *tet* genes had MICs of ≥132 μg/ml. Bryan et al. (7) observed an MIC of ≥93 μg/ml in *E. coli* isolates from humans and animals associated with one or more tetracycline resistance genes.

The majority of the isolates (85.3%) we screened were highly susceptible to ceftiofur. Choi et al. (9) and Hariharan et al. (17) made similar observations for pathogenic *E. coli* isolates from swine and calves. *Escherichia coli* isolates that grew at ≥8 μg/ml have been isolated from retail meat by Zhao et al. (57) and Schroeder et al. (49). Three *E. coli* isolates had MICs of ≥128 μg/ml for ceftiofur in our study. Similar MICs have been reported for *E. coli* isolates from calf scours, feces of cattle, and carcass swabs (6, 50). Resistance to ceftiofur, ampicillin, and the beta-lactamase inhibitor clavulanic acid in *E. coli* is associated primarily with the AmpC class of cephamycinases (bla_{CMY}) (57). The multidrug resistance of ceftiofur-resistant *E. coli* isolates to other unrelated antimicrobials such as tetracycline and chloramphenicol observed in our study has previously been documented in *Salmonella* and *E. coli* isolated from animals (55, 54).

Blake et al. (5) observed that porcine *E. coli* isolates that are resistant to tetracycline also exhibited resistance to ampicillin and streptomycin, often in combination with chloramphenicol. Multidrug resistance patterns in our study included resistance to the above-mentioned antimicrobials. White et al. (54) reported similar multidrug resistance in *E. coli* isolates from bovine diarrhea that were selected for chloramphenicol and florfenicol resistance.

We used PFGE to study tetracycline-resistant *E. coli* isolates ($n = 99$) and found 60 subtypes. Other studies have reported similar levels of high diversity. Subtypes of *E. coli* isolated from sewage, gulls, and dairy cattle exhibited extreme diversity (4, 34). Even with the amount of diversity observed within a relatively small sample size (average, one to two isolates per cow), we were able to isolate the same clonal type from different cows repeatedly. Interestingly, each farm had a reservoir of *E. coli* clonal types that circulated within the animals of the farm but were distinct from isolates from other farms. Further studies are needed to understand the scope of diversity in *E. coli* to clarify whether each farm harbors a unique set of genotypes or whether our results are confounded due to small sampling size.

To understand the wide spread of tetracycline resistance among subtypes of *E. coli*, isolates were screened for *tet* determinants. The predominance of the *tet*(B) efflux gene observed in our study has previously been documented in coliforms (73%) of human and animal origins by Marshall et al. (32). Schnabel and Jones (48) showed that *tet* efflux determinants were carried predominantly by plasmids and that *tet*(A), *tet*(B), and *tet*(C) efflux determinants were associated with transposons. Lee et al. (28) observed that in *E. coli* isolates from domestic pigs, *tet*(B) and *tet*(A) were carried on plasmids. Others have shown that *tet* efflux genes are part of plasmid-borne transposons in gram-negative bacteria (21, 36). The wide distribution of *tet*(B) across gram-negative genera, including *Escherichia*, *Enterobacter*, *Proteus*, *Salmonella*, *Actinobacillus*, *Haemophilus*, *Moraxella*, and *Treponema* spp., indicates that it is highly likely that horizontal transfer of tetracycline resistance occurs (10, 43, 52). Conjugative plasmids have undoubtedly contributed to the spread of efflux gene classes A to E within gram-negative bacteria and classes K and L within gram-positive bacteria (27). The majority of gram-negative efflux genes are normally associated with large plasmids, most of which are conjugative and belong to different incompatibility groups (21, 36). The presence of *tet*(A) and *tet*(B) on the

chromosome, rather than on a plasmid, as frequently reported in the literature, was an intriguing observation in our study.

In summary, we found tetracycline- and ampicillin-resistant GN-EB in the feces of healthy lactating dairy cattle. Nine bacterial species were identified from MAC plates supplemented with four commonly used antimicrobial agents, of which *E. coli* was the most frequently isolated organism. The MICs for *E. coli* were comparable to those for *E. coli* isolated from clinical and food samples reported previously. The majority of the tetracycline-resistant *E. coli* isolates encoded the *tet*(B) determinant, which was determined to be located on the chromosome and not on plasmids, as reported by other researchers. PFGE analysis revealed that tetracycline-resistant *E. coli* isolates were highly diverse and, on most occasions, were localized to a given dairy herd. Routine screening of commensal GN-EB, in particular *E. coli*, could facilitate the study of current and prospective concerns related to the emergence of antimicrobial-resistant GN-EB in dairy cattle.

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