Applied and Environmental Microbiology, June 2008, p. 3658–3666 Vol. 74, No. 12
0099-2240/08/$08.00+0 doi:10.1128/AEM.02505-07


# Associations between Antimicrobial Resistance Genes in Fecal Generic *Escherichia coli* Isolates from Cow-Calf Herds in Western Canada[▽]

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Received 6 November 2007/Accepted 11 April 2008

**The objective of this study was to examine associations among the genetic determinants of antimicrobial resistance (AMR) in 207 fecal generic *Escherichia coli* isolates obtained from 77 cow-calf herds in western Canada. Twenty-three resistance genes corresponding to six different antimicrobial families were assessed using DNA hybridization and PCR. The most common resistance genes in the study sample (207 isolates) were *sul2* (48.3%), *tet*(B) (45.4%), and *ant(3″)-Ia* (*aadA1*) (19.3%). Several statistically significant associations between the examined resistance genes were detected. The strongest associations observed were those between genes for resistance to chloramphenicol (*catI*) and trimethoprim (*dhfrI*) (odds ratio [OR] = 214; $P = 0.0001$), sulfonamide (*sul1*) and chloramphenicol (*catI*) (OR = 96.9; $P = 0.0001$), streptomycin [*ant(3″)-Ia* (*aadA1*)] and trimethoprim (*dhfrI*) (OR = 96.2; $P = 0.0001$), sulfonamide (*sul1*) and streptomycin [*ant(3″)-Ia* (*aadA1*)] (OR = 79.3; $P = 0.0001$), and tetracycline [*tet*(B)] and sulfonamides (*sul2*) (OR = 25.7; $P = 0.0001$). At least one of the resistance genes corresponding to each nonaminoglycoside family of antimicrobials examined in this study was associated with the two aminoglycoside resistance genes *ant(3″)-Ia* (*aadA1*) and *aph(3′)-Ia*. The multiple, strong associations between genes and the diverse nature of the associations described in this study demonstrate the complexity of resistance gene selection in cow-calf herds and should be considered in the planning of AMR control practices for cow-calf operations.**

Antimicrobial resistance (AMR) is an important issue in both human and veterinary medicine. Understandably, many studies have focused on organisms that are pathogenic for people, including *Salmonella* spp. (46), *Campylobacter* spp. (12), and *Escherichia coli* O157 (28). However, transmissible genetic elements encoding AMR can also be maintained in commensal bacteria (10, 38, 41), and resistance gene transmission from nonpathogenic to pathogenic organisms within the intestinal tract may be important for the development of AMR (45). *E. coli* has developed a number of elaborate methods for acquiring and disseminating genetic determinants and may serve as a reservoir for transmissible resistance (31). Studying the molecular determinants of resistance in generic *E. coli* isolates will increase our understanding of the significance of commensal bacteria in the development and transfer of AMR.

Antimicrobial use (AMU) has an impact on the distribution of AMR phenotypes (1, 13, 27) and resistance genes (4). As a result of the variety of AMU practices for different livestock species, AMR phenotypes and genotypes in one livestock class may not be representative of those in another. Therefore, describing AMR throughout all phases of livestock production is vital to understanding the epidemiology of AMR. To date, there are little information available on AMR in cow-calf herds and no data for western Canada, although the western provinces of Alberta and Saskatchewan contain more than 65% of the beef cow, breeding heifer, and calf populations in Canada (42).

The spread of mobile genetic elements such as plasmids, transposons, and integrons or gene cassettes (3, 18, 40) may be responsible for the rapid dissemination of multiple AMR genes (22, 36, 39) and the exchange of resistance genes between pathogens and nonpathogens or between gram-positive and gram-negative bacteria (33, 35). AMR genes can aggregate on a single mobile element in such a way that antimicrobials of a different class or even nonantibiotic substances like heavy metals or disinfectants can select for bacteria resistant to therapeutic antimicrobial agents (34, 35). Since resistance genes can be linked on mobile genetic elements, the use of a particular antimicrobial can select for resistance not only to that antimicrobial but also potentially to a variety of others. This pattern means that even if there is restricted use of certain antimicrobials, the resistance genes associated with these restricted antimicrobials may still be perpetuated through coselection. An understanding of the associations between resistance genes may have important implications for the design of effective prudent-use guidelines. The objective of this study was to describe the associations between genetic determinants of AMR in fecal generic *E. coli* isolates obtained from cow-calf herds in western Canada.

## MATERIALS AND METHODS

**General aspects of the study and sample collection.** Fecal samples were collected from 1,407 individually identified animals on 148 privately owned

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▽ Published ahead of print on 18 April 2008.

TABLE 1. MIC distribution for 207 isolates tested for antimicrobial susceptibilities by using the Sensititre 2002 NARMS CMV7CNCD plate configuration

| Ranking[a] | Antimicrobial | MIC (μg/ml) | | Range of concns (μg/ml) tested | % of isolates[b] for which the MIC (μg/ml) was: | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Median | 75th percentile | | ≤0.015 | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | >512 |
| I | Ceftiofur | 0.25 | 0.25 | 0.12–8 | | | | 2.4 | 72.9 | 20.8 | 1.0 | 0.5 | 1.0‖ | 1.0 | **0.5** | | | | | | |
| | Ceftriaxone | ≤0.25 | 0.25 | 0.25–64 | | | | | 96.1 | 1.4 | | ‖ | 1.0 | 1.0 | 0.5 | ‖ | | | | | |
| | Ciprofloxacin | ≤0.015 | ≤0.015 | ≤0.015–4 | 100.0 | | | | | | | | | | | | | | | | |
| II | Amikacin | 2 | 2 | 0.5–4 | | | | | | 0.5 | 33.8 | 61.4 | 3.9 | **0.5** | ‖ | | | | | | |
| | Amoxicillin-clavulanic acid | 4 | 4 | 1–32 | | | | | | | 0.5 | 25.6 | 53.6 | 12.6 | 2.9‖ | 2.4 | **2.4** | | | | |
| | Gentamicin | 1 | 1 | 0.25–32 | | | | 18.4 | 28.5 | 51.2 | 1.0 | | ‖ | 0.5 | **0.5** | | | | | | |
| | Kanamycin | ≤8 | ≤8 | 8–64 | | | | | | | | 42.5 | 85.0 | ‖ | ‖ | | **15.0** | | | | |
| | Nalidixic acid | 4 | 4 | 0.5–32 | | | | | | | | | 57.5 | | | | | | | | |
| | Streptomycin | ≤32 | 64 | 32–64 | | | | | | | | | | | 58.5‖ | 26.1 | **15.5** | | | | |
| | Trimethoprim-sulfamethoxazole | 0.25 | 0.5 | 0.12–4 | | | | 49.8 | 19.8 | 14.0 | 0.5 | ‖ | | **15.9** | | | | | | | |
| III | Ampicillin | 4 | 4 | 1–32 | | | | | | 3.4 | 35.7 | 39.6 | 2.4 | 0.5‖ | 0.5 | **17.9** | | | | | |
| | Cefoxitin | 4 | 4 | 0.5–16 | | | | | | | 15.0 | 64.7 | 15.0 | 0.5‖ | **4.8** | | | | | | |
| | Cephalothin | 8 | 16 | 2–32 | | | | | | | | 16.9 | 53.6 | 24.6‖ | 1.0 | **3.9** | | | | | |
| | Chloramphenicol | 4 | 8 | 2–32 | | | | | | | 3.9 | 46.9 | 33.8 | 1.0‖ | | **14.5** | | | | | |
| | Sulfamethoxazole | >512 | >512 | 16–512 | | | | | | | | | | 44.9 | | | | | | | |
| | Tetracycline | ≥64 | ≥64 | 4–32 | | | | | | | | 40.1 | 1.0‖ | 1.9 | 1.0 | **56.0** | | | ‖ | 1.9 | **53.1** |

[a] Rankings I to III are in terms of human importance according to the Veterinary Drugs Directorate, Health Canada.

[b] Numbers are percentages of the total number of isolates. Vertical double bars indicate the breakpoints used for categorization as susceptible and resistant (e.g., a double bar in the 4-μg/ml column indicates a cutoff between 4 and 8 μg/ml). Underlining indicates the percentage of isolates corresponding to the median MIC. Numbers in bold are the percentages of isolates showing growth in all wells within the tested range of concentrations, indicating that the actual MIC was greater than the highest concentration tested.

beef farms in Alberta and Saskatchewan (16, 17). The farms were part of a larger survey for risk factors affecting calf health. Samples were collected from three study groups: 480 calves (group 1) and 533 cows (group 2) sampled in the spring of 2002 and 394 calves (group 3) sampled in the fall of 2002. The spring samples (obtained during the period from March to July) were collected from accessible cows or calves in the calving and nursery area. The fall samples (obtained during the period from August to December) were collected from calves prior to weaning and during fall processing. Fecal samples were obtained either directly from the rectum or from the ground immediately after defecation. A separate disposable glove and a separate container were used for each animal.

***E. coli* culture.** Fecal samples on ice were sent for culture at the Prairie Diagnostic Services, Saskatoon, Saskatchewan, Canada. The samples were cultured on MacConkey agar plates at 37°C for 18 h for the isolation of *E. coli*. At least three individual lactose-fermenting colonies from each sample were identified as *E. coli* by using standard biochemical tests, including indole, triple sugar iron slant, citrate, and urea tests. If both dry and mucoid colonies within a sample were detected, then three isolates from each colony type were tested. Individual *E. coli* isolates were stored in a solution of 50% glycerol and Luria-Bertani broth at −80°C until susceptibility testing was completed.

**Susceptibility testing methodology.** *E. coli* isolates were tested for susceptibility by Agri-Food Laboratories Branch, Alberta, Agriculture and Food, Edmonton, Alberta, Canada, by using a broth microdilution technique for 16 antimicrobials (Sensititre; TREK Diagnostic Systems Inc., Cleveland, OH) and the standard 2002 National Antimicrobial Resistance Monitoring System (NARMS) panel (Table 1). Breakpoints for susceptibility as defined by the Clinical and Laboratory Standards Institute (CLSI) were used (29, 30). All isolates that fell into the intermediate-susceptibility range were classified as susceptible. Isolates for which amikacin MICs were ≤4 μg/ml were classified as susceptible, and the results for isolates for which MICs were >4 μg/ml were considered not interpretable because the breakpoint is 4 dilutions beyond the range tested for the panel. The breakpoint used for streptomycin was 64 μg/ml. This breakpoint is based on data from the NARMS because no CLSI *Enterobacteriaceae* interpretative criteria for streptomycin are available (7).

**Selection of samples for genotype testing.** Genetic testing was completed for 12.2% (134 of 1,099) of all resistant isolates collected and 2.2% (73 of 3,319) of all susceptible isolates. This subset of 207 isolates was selected using a random number generator and was stratified based on the resistance status and the study group. Only one isolate per animal was included; therefore, this subset of isolates represents 207 animals from 77 farms.

**Genotyping.** DNA hybridization was used to test for 23 resistance genes corresponding to six antimicrobial families. Table 2 lists the genetic markers examined according to antimicrobial family, identifies the sources providing the DNA, and gives the PCR primer sequences used to generate DNA hybridization probes and confirm hybridization results by PCR.

The 28 strains used as positive controls and templates for DNA amplification were obtained from different laboratories (25, 26). These strains were stored at −80°C in tryptic soy broth medium containing 10% glycerol (vol/vol) and were propagated on Luria-Bertani broth or agar containing one of the following antimicrobial agents at the appropriate concentration: ampicillin (50 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), trimethoprim (10 μg/ml), and sulfamethazine (200 μg/ml).

Oligonucleotide primers for PCR amplification of AMR gene sequences have been described by Maynard et al. (25, 26). Template DNA from bacterial cultures was prepared by the boiling method of Daigle et al. (8). PCR mixtures (total volume, 50 μl) contained 1× PCR buffer (10 mM Tris-HCl, pH 9.0; 50 mM KCl; 1.5 mM $MgCl_2$ [Amersham Pharmacia Biotech Inc., Piscataway, NJ]), 200 μM concentrations of each of the four deoxynucleoside triphosphates, 1 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech Inc.), 25 pmol of each primer, and 5 μl of the template. DNA amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, CA) under the following conditions: 5 min at 94°C, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. An aliquot (3 μl) of each PCR mixture was resolved on a 1.2% agarose gel to confirm the product size and purity. PCR products were labeled with [α-$^{32}$P]dCTP by using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech Inc.). Colony hybridizations were performed as described previously (19).

**Statistical analysis.** Descriptive analyses were completed and variables were recoded as necessary for statistical modeling by using commercially available software programs (SPSS 11.0 for Windows; SPSS Inc., Chicago, IL). Initially, all isolates were coded according to the presence or absence of each resistance phenotype and resistance gene considered in the analysis. Multiple-AMR was defined as phenotypic resistance to ≥2 antimicrobials, and a multiple-resistance-

TABLE 2. Antimicrobial families, genetic markers, primer sequences, GenBank accession numbers, and sources providing DNA for resistance genes tested

| Antimicrobial family | Genetic marker | PCR primer sequence (5′–3′) | | Amplicon size (bp) | GenBank accession no. | Source providing DNA |
|---|---|---|---|---|---|---|
| | | Forward | Reverse | | | |
| Beta-lactams | $bla_{TEM}$ | GAGTATTCAACATTTTCGT | ACCAATGCTTAATCAGTGA | 857 | AF309824 | R. C. Levesque |
| | $bla_{SHV}$ | TCGCCTGTGTATTATCTCCC | CGCAGATAAATCACCACAATG | 768 | AF148850 | R. C. Levesque |
| Aminoglycosides | *aac(3)-IIa* (*aacC2*) | CGGAAGGCAATAACGGAG | TCGAACAGGTAGCACTGAG | 740 | X54723 | D. Sandvang |
| | *aac(3)-IV* | GTGTGCTGCTGGTCCACAGC | AGTTGACCCAGGGCTGTCGC | 627 | X01385 | J. Harel |
| | *aph(3′)-Ia* (*aphA1*) | ATGGGCTCGCGATAATGTC | CTCACCGAGGCAGTTCCAT | 600 | M18329 | J. Harel |
| | *aph(3′)-IIa* (*aphA2*) | GAACAAGATGGATTGCACGC | GCTCTTCAGCAATATCACGG | 680 | V00618 | J. Harel |
| | *ant(3″)-Ia* (*aadAI*) | CATCATGAGGGAAGCGGTG | GACTACCTTGGTGATCTCG | 786 | DQ166553.1 | J. Harel |
| | *ant(3″)-If* (*aadA6*) | GAGTAACGCAGTACCCGC | CACTGGCATGGCACTAAGC | 795 | AY444814.1 | J. Harel |
| Tetracycline | *tet*(A) | GTGAAACCCAACATACCCC | GAAGGCAAGCAGGATGTAG | 888 | X00006 | J. Harel |
| | *tet*(B) | CCTTATCATGCCAGTCTTGC | ACTGCCGTTTTTTCGCC | 774 | J01830 | J. Harel |
| | *tet*(C) | ACTTGGAGCCACTATCGAC | CTACAATCCATGCCAACCC | 881 | J01749 | J. Harel |
| Phenicols | *catI* | AGTTGCTCAATGTACCTAT AACC | TTGTAATTCATTAAGCATTC TGCC | 547 | M62822 | J. Harel |
| | *floR* | CGCCGTCATTCCTCACCTTC | GATCACGGGCCACGCTGTGTC | 215 | AF252855 | D. G. White |
| Trimethoprim | *dhfrI* | AAGAATGGAGTTATCGGG AATG | GGGTAAAAACTGGCCTAAA ATTG | 391 | X00926 | J. Harel |
| | *dhfrIb* | AGTATCATTGATAGCTGCG | GTAGTGCGCGAAGCGAAC | 517 | DQ388123.1 | J. Harel |
| | *dhfrV* | CTGCAAAAGCGAAAAACGG | AGCAATAGTTAATGTTTGAGC TAAAG | 432 | X12868 | O. Sköld |
| | *dhfrVII* | GGTAATGGCCCTGATATCCC | TGTAGATTTGACCGCCACC | 265 | X58425 | O. Sköld |
| | *dhfrIX* | TCTAAACATGATTGTCGC TGTC | TTGTTTTCAGTAATGGTCGGG | 462 | X57730 | C. Wallen |
| | *dhfrXII* | GAACTCGGAATCAGTACGC | ACGCGCATAAACGGAGTG | 483 | DQ157751.1 | J. Harel |
| | *dhfrXIII* | CAGGTGAGCAGAAGATTTTT | CCTCAAAGGTTTGATGTACC | 294 | Z50802 | P. V. Adrian |
| | *dhfrXV* | GGGAACAATTACTCTTC | GTCTTCAGATGATTTAGC | 186 | Z83311D | P. V. Adrian |
| Sulfonamides | *sul1* | TTCGGCATTCTGAATCTCAC | ATGATCTAACCCTCGGTCTC | 822 | X12869 | R. C. Levesque |
| | *sul2* | CGGCATCGTCAACATAACC | GTGTGCGGATGAAGTCAG | 722 | M36657 | J. Harel |

gene pattern was defined as the presence of ≥2 resistance genes in an isolate. Isolates were further categorized by the presence or absence of at least one gene corresponding to each of the six families of antimicrobials considered in this study. For example, if an isolate contained any individual *tet* resistance gene or any combination of *tet* resistance genes, it was classified as being positive for the appropriate individual genes but also as being tetracycline resistance gene positive.

The primary outcome and response variables of interest included the individual resistance genes with prevalence rates of greater than 1.5% (Table 3). Associations between specific resistance genes and their corresponding phenotypes were also examined. Generalized estimating equations and SAS version 8.2 for Windows (PROC GENMOD; SAS Institute, Cary, NC) were used to account for the clustering of isolates collected from a single herd. Model specifications included a binomial distribution, a logit link function, a repeated statement with a subject equal to herd, and an exchangeable correlation structure. Statistically significant associations were reported as odds ratios (OR) with 95% confidence intervals (CI). An OR of >1 indicated the increasing occurrence of the genotype being studied with the increased occurrence of a second measured genotype (a positive association), while an OR of <1 indicated the decreasing occurrence of the genotype being studied with increases in the occurrence of a second measured genotype (a negative association). Multiple comparisons were accounted for by using a Bonferroni correction to provide a conservative estimate for the level of statistical significance (9). An association was significant if the $P$ value was <0.004 after correction for the 12 comparisons made between each outcome and other genotypes ($P < 0.05/k$, where $k$ represents the number of comparisons) (9).

## RESULTS

**Description of sample population for genotyping study.** The selected isolates collected in the spring of 2002 ($n = 107$) were from calves that ranged in age from 1 to 120 days, with a median age of 6 days. The ages of the dams of these calves ranged from 2 to 13 years, with a median cow age of 5 years. Fifty-seven percent of the calves were male, and only 8.8% (9 of 107) of the calves in the sample group had clinical signs of disease. Fifty of the 207 selected isolates were from healthy cows that ranged in age from 2 to 10 years, with a median age of 5 years. The final 50 isolates were chosen from samples obtained from calves in the fall (median age of calves, 204 days; range, 118 to 301 days). The median age of the dams of these calves was 6 years (range, 2 to 14 years). Fifty-six percent of these calves were male, and all the calves were classified as healthy.

**Phenotypic antimicrobial susceptibilities of the selected isolates.** Sixty-five percent of the 207 selected isolates were resistant to at least one antimicrobial (Table 4). The antimicrobials to which resistance was most commonly detected were tetracycline, sulfamethoxazole, and streptomycin (Table 4). No isolates were resistant to ceftriaxone, ciprofloxacin, or nalidixic acid.

Twenty-nine different patterns of multiple-AMR (resistance to ≥2 antimicrobials) were detected. The most common pattern (frequency, 17.9%; found in 37 of 207 isolates) consisted of resistance to streptomycin, sulfamethoxazole, and tetracycline. The next most common multiple-AMR pattern was resistance to sulfamethoxazole and tetracycline (frequency,

TABLE 3. Antimicrobial agents and associated resistance genes investigated and resistance gene prevalence rates among 207 isolates from beef cattle

| Antimicrobial(s) | Resistance gene | Proportion (%) of isolates (no. of isolates) carrying resistance gene among: | | | |
|---|---|---|---|---|---|
| | | All isolates ($n$ = 207) | Isolates from spring calves ($n$ = 100) | Isolates from fall calves ($n$ = 50) | Isolates from cows ($n$ = 50) |
| Ampicillin | $bla_{TEM}$ | 17.9 (37) | 32.7 (35) | 2.0 (1) | 2.0 (1) |
| | $bla_{SHV}$ | 0 | 0 | 0 | 0 |
| Gentamicin | *aac(3)-IV* | 0.5 (1) | 1.0 (1) | 0 | 0 |
| | *ant(2″)-Ia* | 0 | 0 | 0 | 0 |
| | *aac(3)-IIa* | 0 | 0 | 0 | 0 |
| Neomycin and kanamycin | *aph(3′)-Ia* | 17.9 (37) | 33.6 (36) | 2.0 (1) | 0 |
| | *aph(3″)-IIa* | 0 | 0 | 0 | 0 |
| Streptomycin and spectinomycin | *ant(3″)-Ia* (*aadA1*) | 19.3 (40) | 34.6 (37) | 6.0 (3) | 0 |
| | *ant(3″)-If* (*aadA6*) | 1.5 (3) | 0 | 6.0 (3) | 0 |
| Tetracycline | *tet*(A) | 13.0 (27) | 17.8 (19) | 16.0 (8) | 0 |
| | *tet*(B) | 45.4 (94) | 72.9 (78) | 30.0 (15) | 2.0 (1) |
| | *tet*(C) | 8.7 (18) | 2.8 (3) | 26 (13) | 4.0 (2) |
| Chloramphenicol | *catI* | 13.0 (27) | 24.3 (26) | 2.0 (1) | 0 |
| | *floR* | 3.4 (7) | 4.7 (5) | 4.0 (2) | 0 |
| Trimethoprim | *dhfrI* | 16.9 (35) | 30.8 (33) | 4.0 (2) | 0 |
| | *dhfrIb* | 1.0 (2) | 1.9 (2) | 0 | 0 |
| | *dhfrV* | 1.0 (2) | 1.9 (2) | 0 | 0 |
| | *dhfrVII* | 0.5 (1) | 1.0 (1) | 0 | 0 |
| | *dhfrIX* | 0.5 (1) | 0 | 0 | 2.0 (1) |
| | *dhfrXII* | 1.9 (4) | 3.8 (4) | 0 | 0 |
| | *dhfrXIII* | 0.5 (1) | 1.0 (1) | 0 | 0 |
| | *dhfrXV* | 0 | 0 | 0 | 0 |
| Sulfonamides | *sul1* | 14.5 (30) | 25.3 (27) | 6.0 (3) | 0 |
| | *sul2* | 48.3 (100) | 74.8 (80) | 38.0 (19) | 2.0 (1) |
| Total % (no.) of isolates positive for any resistance gene | | 64.3 (133) | 89.7 (96) | 64.0 (32) | 10.0 (5) |

10.6%; found in 22 of 207 isolates). The median number of antimicrobials per resistance pattern was 3, and the maximum was 11.

**Resistance genes detected in the selected isolates.** Resistance genes were detected in 64.3% of the 207 isolates tested, in 93.3% (125 of 134) of resistant isolates, and in 11.0% (8 of 73) of susceptible isolates. The most commonly detected genes included a marker for sulfonamide resistance, *sul2*; one for tetracycline resistance, *tet*(B); and a gene for streptomycin resistance, *ant(3″)-Ia* (also called *aadA1*) (Table 3). The resistance genes $bla_{SHV}$, *ant(2″)-Ia*, *aac(3)-IIa*, *aph(3″)-IIa*, and *dhfrXV* were not detected in any isolates.

Sixteen different patterns of multiple resistance genes (≥2 resistance genes) were identified. The median number of resistance genes in the observed patterns was two, with a maximum of seven. The most common pattern detected consisted of *sul2* and *tet*(B) together (found in 40 isolates [19.3%]). The next most common pattern (found in six isolates [2.9%]) consisted of $bla_{TEM}$, *aph(3′)-Ia*, *tet*(B), and *sul2*.

**Phenotypic antimicrobial susceptibilities and associated resistance genes.** Genotypes did not always correspond with the phenotypic expression within individual isolates. Eight isolates carried resistance genes but had no evidence of phenotypic resistance (Table 5). Conversely, there were also eight isolates that were classified as resistant based on MICs but had no identified resistance genes. Of these, six were resistant to sulfamethoxazole; one was resistant to amoxicillin-clavulanic acid, ampicillin, and cefoxitin; and one was resistant to streptomycin, tetracycline, and sulfamethoxazole.

There were 122 isolates classified as resistant to tetracycline based on the MICs; of these isolates, 15 (12%) had *tet*(A), 83 (68%) had *tet*(B), 4 (3.3%) had *tet*(C), 3 (2.5%) had *tet*(A) and *tet*(B) together, 7 (5.7%) had *tet*(A) and *tet*(C) together, and 1 isolate (0.8%) was *tet*(A), *tet*(B), and *tet*(C) positive. There were also 9 isolates (7.4%) that were classified as resistant to tetracycline that did not have a corresponding tetracycline resistance gene. Tetracycline resistance was not associated with the presence of the *tet*(A) or *tet*(C) gene but was strongly associated with the presence of *tet*(B). Isolates that were positive for tetracycline resistance were 29.2 times (95% CI, 11.1 to 76.6; $P = 0.0001$) more likely to be positive for *tet*(B) than isolates that were tetracycline susceptible.

Of the 114 sulfamethoxazole-resistant isolates, 9 (7.9%) had *sul1*, 76 (67%) had *sul2*, 20 (18%) had *sul1* and *sul2* together, and 9 (7.9%) were positive for sulfamethoxazole resistance phenotypically but not genotypically. Of the trimethoprim-sul-

TABLE 4. Antimicrobial agents investigated and resistance phenotype prevalence rates among 207 isolates from beef cattle

| Antimicrobial(s) | Proportion (%) of isolates (no. of isolates) showing resistance to antimicrobial(s) or carrying resistance gene among: | | | |
|---|---|---|---|---|
| | All isolates ($n$ = 207) | Isolates from spring calves ($n$ = 100) | Isolates from fall calves ($n$ = 50) | Isolates from cows ($n$ = 50) |
| Amikacin | NI[a] | NI | NI | NI |
| Amoxicillin-clavulanic acid | 4.8 (10) | 6.5 (7) | 6.0 (3) | 0 |
| Ampicillin | 18.4 (38) | 32.7 (35) | 6.0 (3) | 0 |
| Cefoxitin | 4.8 (10) | 6.5 (7) | 6.0 (3) | 0 |
| Ceftiofur | 1.5 (3) | 1.9 (2) | 2.0 (1) | 0 |
| Ceftriaxone | 0 | 0 | 0 | 0 |
| Cephalothin | 4.8 (10) | 7.5 (8) | 4.0 (2) | 0 |
| Gentamicin | 1.0 (2) | 1.9 (2) | 0 | 0 |
| Kanamycin | 15.0 (31) | 28.0 (30) | 2.0 (1) | 0 |
| Streptomycin | 41.6 (86) | 67.3 (72) | 28.0 (14) | 0 |
| Chloramphenicol | 14.5 (30) | 25.3 (27) | 6.0 (3) | 0 |
| Ciprofloxacin | 0 | 0 | 0 | 0 |
| Nalidixic acid | 0 | 0 | 0 | 0 |
| Sulfamethoxazole | 55.1 (114) | 81.3 (87) | 52.0 (26) | 2.0 (1) |
| Tetracycline | 58.9 (122) | 87.0 (93) | 56.0 (28) | 2.0 (1) |
| Trimethoprim-sulfamethoxazole | 15.9 (33) | 29.0 (31) | 4.0 (2) | 0 |
| Total % (no.) of isolates positive for AMR | 64.7 (134) | 87.6 (94) | 78.0 (39) | 2.0 (1) |

[a] NI, result not interpretable.

famethoxazole-resistant isolates ($n$ = 33), 27 (81.8%) had *dhfrI*, 1 (3.0%) had *dhfrVII*, 2 (6.1%) had *dhfrXII*, 1 (3.0%) had *dhfrXII* and *dhfrXIII* together, and 2 (6.1%) had *dhfrIb* and *dfhrV* together. Sixty-seven (58.8%) of the 114 sulfamethoxazole-resistant isolates were positive for at least one sulfonamide resistance gene, 5 (4.4%) were positive for at least one sulfonamide resistance gene and one trimethoprim resistance gene, and 33 (28.9%) were positive for at least one sulfonamide resistance gene and one trimethoprim resistance gene and were also phenotypically resistant to trimethoprim-sulfamethoxazole. Sulfamethoxazole resistance was associated with both *sul1* and *sul2*, but the association with *sul2* was much stronger. Sulfamethoxazole-resistant isolates were 33.8 times (95% CI, 3.2 to 356.4; $P$ = 0.003) and 125.4 times (95% CI, 41.5 to 378.4; $P$ = 0.0001) more likely to be positive for the resistance genes *sul1* and *sul2*, respectively, than sulfamethoxazole-susceptible isolates. Trimethoprim-sulfamethoxazole resistance was associated with *dhfrI* but not *dhfrXII*. Trimethoprim-sulfamethoxazole-resistant isolates were 121.3 times (95% CI, 30.6 to 480.7; $P$ = 0.0001) more likely to be *dhfrI* positive than trimethoprim-sulfamethoxazole-susceptible isolates.

TABLE 5. Resistance genes present in isolates with susceptible phenotypes ($n$ = 8)[a]

| Resistance gene(s) present | No. of isolates with gene pattern and no resistance detected |
|---|---|
| *aph(3′)-Ia*, *tet*(B), *sul2* | 1 |
| $bla_{TEM}$ | 1 |
| $bla_{TEM}$, *aph(3′)-Ia*, *ant(3″)-Ia* (*aadA1*), *dhfrI*, *dhfrXII*, *sul2* | 1 |
| $bla_{TEM}$, *tet*(B) | 1 |
| *dhfrIX* | 1 |
| *tet*(A), *tet*(C) | 1 |
| *tet*(C) | 2 |
| Total no. of resistance gene-positive isolates with no resistance detected | 8 |

[a] None of the indicated isolates showed any resistance phenotype.

The majority of the chloramphenicol-resistant isolates ($n$ = 30) were *catI* positive (21 isolates [70.0%]), 5 (16.7%) were *floR* positive, and 4 (13.3%) had no corresponding chloramphenicol resistance gene. Chloramphenicol-resistant isolates were 17.5 times (95% CI, 3.5 to 87.4; $P$ = 0.0005) and 63.1 times (95% CI, 18.2 to 218.2; $P$ = 0.0001) more likely to be positive for the resistance genes *floR* and *catI*, respectively, than chloramphenicol-susceptible isolates.

The gentamicin-resistant isolates ($n$ = 2) had resistance genes *aac(3)-IV* and *ant(3″)-Ia* (*aadA1*) together (one isolate) and *ant(3″)-Ia* (*aadA1*) and *aph(3′)-Ia* together (one isolate). Of the 31 kanamycin-resistant isolates, 14 (45.2%) had the *aph(3′)-Ia* resistance gene, 1 (3.2%) had *ant(3″)-Ia* (*aadA1*), 15 (48.3%) had *ant(3″)-Ia* (*aadA1*) and *aph(3′)-Ia* together, and 1 (3.2%) had no associated resistance gene. Almost half of the streptomycin-resistant isolates (41 isolates [47.7%]) did not have an associated streptomycin resistance gene, 14 (16.3%) had the *aph(3′)-Ia* resistance gene, 9 (10.5%) had *ant(3′)-Ia* (*aadA1*), 20 (23.3%) had *ant(3′)-Ia* (*aadA1*) and *aph(3′)-Ia* together, 1 isolate (1.1%) had *ant(3′)-Ia* (*aadA1*) and *ant(3′)-Ia* (*aadA6*), and 1 (1.1%) had *aac(3)-IV* and *ant(3′)-Ia* (*aadA1*) together. Positive associations between resistance to streptomycin and kanamycin and the respective resistance genes, *ant(3″)-Ia* (*aadA1*) and *aph(3′)-Ia*, were detected. Kanamycin- and streptomycin-resistant isolates, respectively, were 6.5 times (95% CI, 3.1 to 13.7; $P$ = 0.0001) and 6.6 times (95% CI, 2.8 to 15.2; $P$ = 0.0001) more likely to be positive for the resistance gene *ant(3″)-Ia* (*aadA1*) than kanamycin- and streptomycin-susceptible isolates. Kanamycin- and streptomycin-resistant isolates, respectively, were 306 times (95% CI, 64.9 to 1440; $P$ = 0.0001) and 26.4 times (95% CI, 8.1 to 86.1;

$P = 0.0001$) more likely to be positive for the resistance gene *aph(3′)-Ia* than kanamycin- and streptomycin-susceptible isolates.

Ampicillin-resistant isolates were 85.8 times (95% CI, 26.8 to 275.2; $P = 0.0001$) more likely to be positive for the resistance gene $bla_{TEM}$ than ampicillin-susceptible isolates.

**Unconditional associations between resistance genes.** Numerous significant associations ($P < 0.004$) between the various individual resistance genes examined were detected (Table 6). The strongest observed associations, ordered by magnitude, included those between the following genes: *catI* and *dhfrI*, *ant(3″)-Ia* (*aadA1*) and *dhfrI*, *sul1* and *catI*, *ant(3″)-Ia* (*aadA1*) and *catI*, *ant(3″)-Ia* (*aadA1*) and *sul1*, *floR* and *dhfrXII*, *tet*(B) and *sul2*, *sul1* and *dhfrI*, and $bla_{TEM}$ and *aph(3′)-Ia*.

Table 7 describes the associations between resistance genes as summarized for each antimicrobial family. At least one resistance gene corresponding to every nonaminoglycoside family of antimicrobials was significantly associated with at least one resistance gene corresponding to the aminoglycoside family.

## DISCUSSION

To gain a better understanding of the epidemiology and the implications of AMR in beef herds, we examined the associations between AMR genes in fecal generic *E. coli* isolates obtained from cow-calf herds. The complex nature of AMR was demonstrated by the large number of significant associations of moderate to substantial magnitude that were detected between resistance genes.

Resistance genes are often associated with integrons or mobile DNA elements such as plasmids and transposons that facilitate the spread of resistance genes (20, 43). The large number of strong associations between genes is consistent with the hypothesis that there is a linkage between many of these resistance genes on mobile elements. The exact mechanisms of association have not been determined in the present study, but previous studies have demonstrated that strong statistical associations between resistance genes can frequently be substantiated by molecular investigations (5, 44).

In this study population, a very strong association between certain phenotypes and genotypes was detected, indicating that the resistance to a given antimicrobial was caused in the majority of cases by a single gene. Examples of such situations include kanamycin and *aph(3′)-Ia*, chloramphenicol and *catI*, sulfamethoxazole and *sul2*, trimethoprim-sulfamethoxazole and *dhfrI*, and ampicillin and $bla_{TEM}$.

In some instances, the phenotype or the genotype alone did not accurately predict the other. Molecular mechanisms underlying AMR are numerous and complex, and the presence or absence of a specific gene corresponding to a particular phenotype does not necessarily imply that the particular strain is resistant or susceptible (2). Resistant phenotypes can emerge from many different genetic determinants, and each determinant may present unique epidemiological features (23). The divergence between genotype and phenotype observed in this study may be simply the result of not testing for all possible resistance genes or of genes' not being turned on in certain isolates. Examples of genes that were not tested for and that may account for the discrepancies between genotypes and phe-

TABLE 6. Statistically significant[a] unconditional associations between antimicrobial resistance genes

| Resistance gene | Associated resistance gene | OR | 95% CI | *P* value |
|---|---|---|---|---|
| *ant(3″)-Ia* (*aadA1*) | $bla_{TEM}$ | 11.5 | 4.9–27.2 | 0.0001 |
| | *catI* | 53.9 | 13.2–221 | 0.0001 |
| | *aph(3′)-Ia* | 10.0 | 4.5–22.4 | 0.0001 |
| | *tet*(A) | 6.2 | 2.3–16.6 | 0.0003 |
| | *su11* | 52.3 | 11.7–233 | 0.0001 |
| | *dhrfI*[b] | 96.2 | 30.2–306 | 0.0001 |
| *aph(3′)-Ia* | $bla_{TEM}$ | 22.7 | 6.6–78.1 | 0.0001 |
| | *catI* | 9.0 | 3.5–22.7 | 0.0001 |
| | *ant(3″)-Ia* (*aadA1*) | 10.7 | 4.7–24.5 | 0.0001 |
| | *tet*(B) | 16.8 | 5.0–56.8 | 0.0001 |
| | *su11* | 5.9 | 2.3–15.1 | 0.0002 |
| | *sul2* | 17.4 | 4.2–71.6 | 0.0001 |
| | *dhfrI* | 18.2 | 7.1–47.2 | 0.0001 |
| *tet*(A) | *ant(3″)-Ia* (*aadA1*) | 6.7 | 2.5–17.9 | 0.0001 |
| | *tet*(B) | 0.2 | 0.1–0.5 | 0.0012 |
| | *tet*(C) | 8.7 | 2.4–30.7 | 0.0008 |
| | *sul1* | 15.0 | 5.6–40.0 | 0.0001 |
| *tet*(B) | *aph(3′)-Ia* | 9.5 | 3.3–27.1 | 0.0001 |
| | *tet*(A) | 0.2 | 0.1–0.5 | 0.0008 |
| | *sul2* | 25.7 | 12.0–54.8 | 0.0001 |
| *tet*(C) | *floR* | 17.8 | 3.9–80.8 | 0.0002 |
| | *tet*(A) | 6.4 | 2.0–20.9 | 0.002 |
| *catI* | $bla_{TEM}$ | 7.0 | 2.8–17.8 | 0.0001 |
| | *aph(3′)-Ia* | 8.9 | 3.6–22.0 | 0.0001 |
| | *ant(3″)-Ia* (*aadA1*) | 56.8 | 12.2–266 | 0.0001 |
| | *sul1* | 83.0 | 21.3–323 | 0.0001 |
| | *dhfrI* | 214 | 46.3–989 | 0.0001 |
| *floR* | *tet*(C) | 17.4 | 3.3–92.4 | 0.0008 |
| | *dhfrXII* | 39.4 | 7.8–200 | 0.0001 |
| *sul1* | $bla_{TEM}$ | 5.0 | 1.9–13.4 | 0.0012 |
| | *catI* | 96.9 | 23.4–401 | 0.0001 |
| | *aph(3′)-Ia* | 6.2 | 2.4–16.4 | 0.0002 |
| | *ant(3″)-Ia* (*aadA1*) | 79.3 | 14.3–441 | 0.0001 |
| | *tet*(A) | 16.1 | 5.4–47.9 | 0.0001 |
| | *dhfrI* | 27.4 | 9.9–75.4 | 0.0001 |
| *sul2* | *aph(3′)-Ia* | 16.5 | 3.1–87.2 | 0.0009 |
| | *tet*(B) | 33.9 | 15.5–74.3 | 0.0001 |
| *dhfrI* | $bla_{TEM}$ | 13.2 | 4.5–39.1 | 0.0001 |
| | *catI* | 193 | 44.6–836 | 0.0001 |
| | *aph(3′)-Ia* | 16.5 | 6.6–40.9 | 0.0001 |
| | *ant(3″)-Ia* (*aadA1*) | 86.4 | 26.3–283 | 0.0001 |
| | *sul1* | 23.2 | 8.4–63.7 | 0.0001 |
| *dhfrXII* | *floR* | 32.4 | 6.5–162 | 0.0001 |
| $bla_{TEM}$ | *catI* | 7.2 | 2.7–18.9 | 0.0001 |
| | *aph(3′)-Ia* | 22.9 | 6.7–77.7 | 0.0001 |
| | *ant(3″)-Ia* (*aadA1*) | 12.3 | 5.1–29.7 | 0.0001 |
| | *sul1* | 4.8 | 1.8–12.4 | 0.0013 |
| | *dhfrI* | 14.6 | 4.7–44.9 | 0.0001 |

[a] Only statistically significant ($P < 0.004$) associations are reported. A total of 207 isolates were examined.

[b] As an example, isolates that were positive for the resistance gene *dhfrI* were 96.2 times (95% CI, 30.2 to 306; $P = 0.0001$) more likely to be positive for *aadA1* than isolates that were *dhfrI* negative.

TABLE 7. Associations between resistance determinants as summarized according to antimicrobial families[a]

| Resistance profile | Association with resistance to: | | | | | |
|---|---|---|---|---|---|---|
| | Aminoglycosides | Tetracycline | Phenicols | Sulfonamide | Trimethoprim | Ampicillin |
| Aminoglycoside resistance | + | | | | | |
| Tetracycline resistance | + | + | | | | |
| Phenicol resistance | + | + | None | | | |
| Sulfonamide resistance | + | + | + | None | | |
| Trimethoprim resistance | + | None | + | + | None | |
| Ampicillin resistance | + | None | + | + | + | NA |

[a] + indicates that an association was detected; "none" indicates that no association was detected. NA, not applicable.

notypes include *strA* and *strB*, *sul3*, *clmA*, and $bla_{CMY-2}$. Untested-for resistance genes were probably involved in the finding of a high percentage of streptomycin-resistant isolates that did not contain a corresponding tested-for resistance gene. Also, given the bimodal distribution of the MICs of ceftriaxone and the distribution of the MICs of ceftiofur, beta-lactam antimicrobial resistance genes not examined in this study may have played a role. The distributions of the MICs of these two antimicrobials suggest acquired resistance in the isolates corresponding to higher values. Additional molecular work would need to be completed to examine the roles of these genes in this population of cow-calf isolates. One other explanation for the discrepancy between phenotypic resistance and the presence of resistance genes may be that the breakpoint may be misplaced, resulting in the misclassification of isolates as susceptible and resistant. Since the breakpoints used for this study are based on data from *E. coli* infections in humans, there may have been a potential for the misclassification of susceptibilities to ceftiofur and ceftriaxone, for which the epidemiological cutoff values are not considered to be identical to the breakpoints used for these antimicrobials. Finally, some resistance phenotypes may be caused by point mutations rather than gene acquisition; therefore, no associated resistance gene would be expected.

The observed associations between resistance genes may be accounted for by AMU practices on cow-calf farms. AMU can drive the selection and coselection of resistance genes. An example of coselection was described by O'Connor et al. (32), who determined that the use of injectable oxytetracycline in cattle receiving chlortetracycline in their feed was associated with an increase in the incidence of resistance to chloramphenicol and sulfasoxazole. An association between *tet*(A) and *sul1* was observed in the present study, which correlates well with the findings of O'Connor and collaborators, although no association between *catI* and any of the tetracycline resistance genes investigated was detected in our study. A potential explanation for the lack of association between tetracycline resistance genes and the chloramphenicol resistance gene *catI* is that the *clmA* gene for chloramphenicol resistance was not considered in this study.

Despite the ban on chloramphenicol use in food-producing animals since 1985 (15), chloramphenicol resistance was detected in the isolates in the present study. This finding may indicate that coselection is contributing to the persistence of chloramphenicol resistance genes in the population (44). In this study, the chloramphenicol resistance gene *catI* was associated with the presence of both a trimethoprim resistance gene (*dhfrI*) and a sulfonamide resistance gene (*sul1*). Therefore, there may be indirect selection for chloramphenicol resistance by the use of trimethoprim or sulfamethoxazole in cow-calf operations. Further characterization of the relationships among the genes and the potential link to AMU should be considered.

There was a negative association between *tet*(A) and *tet*(B) resistance genes among our isolates. An incompatibility of plasmids carrying the tetracycline resistance determinants has been suggested to explain this negative association (21). This may also explain the lack of association between tetracycline resistance and the *tet*(A) resistance gene.

This study did not investigate the presence of class I integrons. Although an integron probe was not included, the *sul1* gene is an integral part of the conserved region of class I integrons. In addition, the *ant(3″)-Ia* (*aadA1*) gene and several different *dhfr* genes have been shown to be present frequently in the variable regions of integrons (6, 11, 14, 24). Despite the lack of testing specifically for integrons, the various patterns consisting of two or more of these genes together with *sul1* suggested the presence of integrons in this sample population.

Several of the associations observed in the present study have also been detected previously in porcine *E. coli* populations (5, 44). These include the integron-related association between *sul1* and *ant(3″)-Ia* (*aadA1*) mentioned above but also the associations between *sul1* and *tet*(A), *sul1* and *catI*, *sul2* and *aphA1*, *ant(3″)-Ia* (*aadA1*) and *aphA1*, and *ant(3″)-Ia* (*aadA1*) and *catI*, as well as the mentioned negative association between *tet*(A) and *tet*(B). Such repeated findings for different animal species and different geographical regions (e.g., cattle from Saskatchewan and Alberta and swine from Ontario [5, 44]) are unlikely to be the result of spurious associations. These results strongly suggest that some mobile genetic elements carrying multiple resistance genes are widespread among farm animal populations or that the AMU patterns are sufficiently consistent across farm animal production systems to result in convergent evolution toward similar gene associations. Only more-detailed molecular characterizations of the genetic elements carrying the implicated genes will allow us to test these two hypotheses.

Associations between indicators of genetic resistance to families of antimicrobials were also examined. While we do recognize, particularly for the aminoglycoside family of antimicrobials, that resistance to one antimicrobial within a family does not confer resistance to the entire family (37), the goal of this investigation was simply to create an initial picture of the complex nature of resistance to the families of antimicrobials

used in cow-calf herds. This exploration provided further insight into the complexity of the epidemiology of AMR.

This network of associations also brings into question the definition of prudent use and the impact of these associations on the development of policy and clinical practice guidelines to minimize AMR. The implication is that current attempts to limit the emergence or spread of AMR based on the careful restriction of the choice of antimicrobials may not prevent selection for genes conferring resistance unrelated to the antimicrobials being used. Therefore, assessments of AMR at the genetic level and of associations between genes are critical tools in devising guidelines for the control of AMR (23). These assessments may be of particular relevance in considering associations between resistance to critically important antimicrobials for therapy for severe infections and resistance to antimicrobials commonly used for prevention and metaphylaxis. The present results describe resistance gene relationships that need to be considered in the planning of potential AMR control measures for cow-calf herds.

## ACKNOWLEDGMENTS

We thank the producers and veterinarians who provided data.

Funding was provided by the Horned Cattle Trust Fund and the Public Health Agency of Canada.

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