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Multiple-Antibiotic Resistance of *Enterococcus faecalis* and *Enterococcus faecium* from Cecal Contents in Broiler Chicken and Turkey Flocks Slaughtered in Canada and Plasmid Colocalization of *tetO* and *ermB* Genes

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ABSTRACT

This study was conducted to characterize the antimicrobial resistance determinants and investigate plasmid colocalization of tetracycline and macrolide genes in *Enterococcus faecalis* and *Enterococcus faecium* from broiler chicken and turkey flocks in Canada. A total of 387 *E. faecalis* and *E. faecium* isolates were recovered from poultry cecal contents from five processing plants. The percentages of resistant *E. faecalis* and *E. faecium* isolates, respectively, were 88.1 and 94% to bacitracin, 0 and 0.9% to chloramphenicol, 0.7 and 14.5% to ciprofloxacin, 72.6 and 80.3% to erythromycin, 3.7 and 41% to flavomycin, 9.6 and 4.3% (high-level resistance) to gentamicin, 25.2 and 17.1% (high-level resistance) to kanamycin, 100 and 94% to lincomycin, 0 and 0% to linezolid, 2.6 and 20.5% to nitrofurantoin, 3 and 27.4% to penicillin, 98.5 and 89.7% to quinupristin-dalfopristin, 7 and 12.8% to salinomycin, 46.7 and 38.5% (high-level resistance) to streptomycin, 95.6 and 89.7% to tetracycline, 73 and 75.2% to tylosin, and 0 and 0% to vancomycin. One predominant multidrug-resistant phenotypic pattern was identified in both *E. faecalis* and *E. faecium* (bacitracin, erythromycin, lincomycin, quinupristin-dalfopristin, tetracycline, and tylosin). These isolates were further examined by PCR and sequencing for the genes encoding their antimicrobial resistance. Various combinations of *vatD, vatE, bcrR, bcrA, bcrB, bcrD, ermB, msrC, linB, tetM,* and *tetO* genes were detected, and *ermB, tetM,* and *bcrB* were the most common antimicrobial resistance genes identified. For the first time, plasmid extraction and hybridization revealed colocalization of *tetO* and *ermB* genes on a ca. 11-kb plasmid in *E. faecalis* isolates, and filter mating experiments demonstrated its transferability. Results indicate that the intestinal enterococci of healthy poultry, which can contaminate poultry meat at slaughter, could be a reservoir for quinupristin-dalfopristin, bacitracin, tetracycline, and macrolide resistance genes.

Antimicrobial resistance is a global issue in both human and veterinary medicine. The presence of antimicrobial-resistant microorganisms in fecal material of animals is becoming a matter of great concern because these microorganisms could be transmitted to humans through a contaminated food supply *(34)*. Studies addressing this concern are mostly conducted on pathogens that pose a direct threat to human health. However, commensal bacteria of the intestinal microbial flora associated with contaminated food are becoming of interest because these commensals can harbor antimicrobial resistance determinants that can spread to animal and/or human pathogens or other commensals *(32, 34)*.

Enterococcus faecium and *Enterococcus faecalis* are part of the normal animal and human gut flora. These bacteria are also ranked among the leading causes of nosocomial infections. Enterococci are ubiquitous in nature and resistant to various environmental conditions. Thus, they have the potential to easily spread through the food chain and contaminate water and the environment *(49)*. These bacteria usually are found in large numbers in food of animal origin, such as cattle, pig, and poultry carcasses *(30, 32),* and their presence is an indication of fecal contamination, which commonly occurs during slaughter of the animals *(32)*. In addition to their intrinsic resistance to many antimicrobials, including resistance to cephalosporins, clindamycin, and low-level resistance to aminoglycosides and other beta-lactams, the enterococci have a remarkable capacity to acquire resistance to other antimicrobials such as ciprofloxacin (CIP), erythromycin (ERY), tetracycline (TET), linezolid (LIZ), daptomycin, quinupristin-dalfopristin (QD), and vancomycin (VAN) *(9, 43, 44)*. Antimicrobial resistance among enterococci is not restricted to nosocomial human settings. Because large amounts of antimicrobial agents are used in animal production, the microbial flora of food animals frequently carries resistance to a range of antimicrobials, including those classified as critically

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important for human therapy by the World Health Organization *(32)*. The results of previous studies have indicated that the use of avoparcin, gentamicin (GEN), and virginiamycin for growth promotion and therapy in food animals has led to the emergence of VAN- and GEN-resistant enterococci and QD-resistant *E. faecium* in animals and meat *(1, 25, 32)*. Consequently, transfer of resistance genes or bacteria from food animals to humans is a potential problem *(32)*. Resistance genes can be localized on plasmids, transposons, or integrons, leading to many possible multiresistance phenotypes and coselection processes. In a recent study, acquired resistance to TET was frequently found in enterococci from poultry carrying the *ermB* gene (macrolide–lincosamide–streptogramin B resistant gene; bacteria are referred to as having MLS_B resistance) *(14)*. However, to our knowledge, plasmid colocalization of *ermB* and *tetO* genes has not been described in enterococci. In Canada, little is known regarding the antimicrobial resistance genes harbored by enterococci of poultry origin.

The purpose of this study was to determine the antimicrobial resistance profiles of *E. faecalis* and *E. faecium* isolates recovered from broiler chicken and turkey flocks slaughtered in Canada and to study plasmid colocalization of *ermB* and *tetO* genes.

MATERIALS AND METHODS

Sample collection and bacterial isolation. Chicken and turkey cecal contents were collected at five processing plants (four chicken and one turkey) located in the province of Quebec, Canada, during 2003 and 2004 as previously described *(10)*. After evisceration, intestines from selected birds were placed into individual sterile plastic bags and kept on melting ice for a maximum of 8 h before culture. For each flock (chicken, $n = 51$; turkey, $n = 40$), two or three pools including cecal contents of approximately 10 birds were created. For each pool, contents were collected from the cecum of each bird using a sterile cotton swab, put in a sterile stomacher bag, and gently manually homogenized. The pooled cecal sample was mixed with 25 ml of buffered peptone water (20 g/liter; Difco, BD, Sparks, MD) until homogenization, and isolation was performed. The contents of the ceca were cultured in enterococcosel broth (Fisher Scientific, Markham, Ontario, Canada), which is a bile esculine azide medium, and incubated at 35°C for 24 h. Ten microliters of culture was then plated on enterococcosel agar (Fisher Scientific) and incubated at 35°C for 24 h. Ten suspected enterococci colonies from each sample were subcultured for purity onto blood agar (tryptic soy agar plus 5% sheep blood). Presumptive identification to the species level was based on positive results for acid production from mannitol, arabinose, and α-methyl-glucoside sugar broths and on the metallic appearance of colonies grown on Slanetz & Bartley medium (Fisher Scientific). Suspected *E. faecalis* and *E. faecium* colonies were selected for multiplex PCR identification to the species level. Strains *E. faecalis* ATCC 29212 and *E. faecium* HA-56038 were used as positive controls.

Bacterial identification. Presumptive biochemically identified *E. faecalis* and *E. faecium* isolates from turkeys and broilers were confirmed by multiplex PCR assay using species-specific primer sets for the *ddl faecalis* (D-alanine–D-alanine ligase gene) and *EM1 faecium* (strongly conserved *E. faecium* genomic DNA fragment) identification genes as previously described *(15, 17)* with minor modifications. DNA extraction was performed with the Chelex 100 (Bio-Rad, Mississauga, Ontario, Canada) ebullition method, in which many loopfuls of pure colonies were mixed with 10% Chelex and boiled for 20 min. The supernatant containing the DNA was used in the multiplex PCR by adding 5 μl of DNA to 2.5 μl of 10× PCR buffer, 0.2 mM concentrations of deoxynucleoside triphosphates (dNTPs), 2 mM $MgCl_2$, 1.25 U of *Taq* DNA polymerase (GE Healthcare, Baie-D'Urfe, Québec, Canada), and 200 mM concentrations of each primer in a total volume of 25 μl. DNA amplification reactions were carried out in a Whatman Biometra thermocycler (Montreal Biotech Inc., Dorval, Québec, Canada) with the following conditions: denaturation for 10 min at 94°C, 30 cycles of 30 s at 94°C, 1 min at 62°C, and 1 min at 72°C, and a final extension for 5 min at 72°C. For visualization of PCR products, 5 μl of each amplicon was subjected to electrophoresis in a 1.7% agarose gel stained with ethidium bromide. A TrackIt 100-bp ladder (Invitrogen, Burlington, Ontario, Canada) was used as the marker. *E. faecalis* MA-58123 and *E. faecium* HA-56038 were used as positive controls.

Antimicrobial susceptibility testing. PCR-confirmed isolates of *E. faecalis* and *E. faecium* were tested for MICs of 17 antibiotics (Tables 1 and 2) according to the recommended Clinical and Laboratory Standards Institute guidelines (M31-A3 and M100-S20) *(20, 21)* (interpretation breakpoints indicated in parentheses): bacitracin (BAC; ≥64 μg/ml), chloramphenicol (CHL; ≥32 μg/ml), CIP (≥4 μg/ml), ERY (≥8 μg/ml), flavomycin (FLA; ≥32 μg/ml), GEN (>500 μg/ml), kanamycin (KAN; ≥1024 μg/ml), lincomycin (LIN; ≥8 μg/ml), LIZ (≥8 μg/ml), nitrofurantoin (NIT; ≥128 μg/ml), penicillin (PEN; ≥16 μg/ml), salinomycin (SAL; ≥8 μg/ml), streptomycin (STR; >1,000 μg/ml), QD (≥4 μg/ml), TET (≥16 μg/ml), tylosin (TYL; ≥32 μg/ml), and VAN (≥32 μg/ml). The broth microdilution method recommended by the National Antimicrobial Resistance Monitoring System (NARMS; plates CMV5ACDC) was used with the Sensititre automated reading and incubation system (ARIS, Trek Diagnostic System Ltd., Cleveland, OH). *Staphylococcus aureus* ATCC 29213 and *E. faecalis* ATCC 29212 and ATCC 51299 were used as quality controls. Breakpoints from NARMS *(7)* were used for BAC, FLA, KAN (high level), and TYL, and breakpoints from DANMAP *(38)* were used for SAL.

Detection of antimicrobial resistance genes and repetitive extragenic palindromic PCR. Detection of antimicrobial resistance determinants was performed using the PCR method with primers and conditions previously described *(2, 11, 18, 23, 26, 35, 41, 45, 47, 48)* with minor modifications. The isolates were examined for the presence of the following genes: *vatD* and *vatE* that encode for streptogramin acetyltransferases; the *bcrABD* operon and its regulatory gene *bcrR* that encode for both a BAC ABC transporter system and an overproduced undecaprenol kinase; *ermB* that encodes a ribosomal methylase that mediates MLS_B resistance; *msrC* that encodes for a macrolide and streptogramin B efflux pump; *linB* that confers resistance to LIN by nucleotidylation; *tetM, tetO,* and *tetS* that encode for TET-minocycline resistance via a ribosomal protection protein mechanism; and *vanA* and *vanB* that encode for inducible VAN resistance via the production of an altered peptidoglycan precursor. The class 1 integron *qacEd1-sul1* gene was determined as previously described *(42)*. Specific PCR assays for detection of *tdn*X and *int* genes also were performed for *tetM*-positive isolates to demonstrate the presence of the Tn*5397*-like and Tn*916*/Tn*1545*-like transposons, respectively *(4, 23)*. A 5-μl aliquot of DNA was added to 2.5 μl of 10× PCR buffer, 0.2 mM

concentrations of dNTPs, 2 mM $MgCl_2$, 150 mM concentrations of each primer (500 mM for *msrC*), and 1.25 U of *Taq* DNA polymerase in a total volume of 25 μl.

New primer constructions (5′ to 3′) were as follows: *vatD* reverse, 5′-GACTTCCTAATGATGCTAT-3′ (position 776, GenBank accession no. L12033); *bcrR* forward, 5′-TATAGGGTTCTCTTGCCGCT-3′, and *bcrR* reverse, 5′-GTTACCCTAACATGGAGTCG-3′ (forward position 3488, reverse position 3913; GenBank accession no. AY496968); *bcrA* forward, 5′-AATCCGTCATGTTGGTAGCTGCTCT-3′, and *bcrA* reverse, 5′-TATTATGCACGAGCCGGAGCTTCT-3′ (forward position 2492, reverse position 2805, GenBank accession no. AY496968); *msrC* forward, 5′-CTTAGGGGTTGCTCAGGAAA-3′, and *msrC* reverse, 5′-AACAAAATCGTTCCCGGATA-3′ (forward position 195, reverse position 392, GenBank accession no. AJ243209); and *vanA* reverse, 5′-ACCTGCAGCGGCCATCATACG-3′ (position 1334, GenBank accession no. 56895).

Positive and negative controls from the strain collection of the Faculty of Veterinary Medicine at the University of Montreal (Québec, Canada) were used in all PCRs. The identity of the gene products of at least one randomly selected isolate for each gene was verified by DNA sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Concord, Ontario, Canada). Repetitive extragenic palindromic (Rep) PCR analysis was performed as previously described *(50)* to address clonality of strains.

Plasmid extraction, hybridization, and transferability. Plasmids from nine selected multiresistant isolates of *E. faecalis* harboring the *tetO* and *ermB* genes were extracted with a Midiprep Kit (Qiagen, Streetsville, Ontario, Canada) according to the manufacturer's specifications with a lysis step of 5 mg/ml lysozyme. Plasmid extracts were subjected to electrophoresis at 100 V for 80 min in a 1% agarose gel stained with ethidium bromide. A supercoiled DNA ladder (Invitrogen) was used as a molecular weight marker. Probes for Southern hybridization were generated by using P^{32}-labeled dNTPs (Perkin-Elmer, Boston, MA) instead of standard dNTPs in the amplification reaction according to the manufacturer's instructions. DNA from plasmid extractions was transferred to positively charged nylon membranes with a vacuum blotter (model 785, Bio-Rad) and probed with P^{32}-labeled PCR products for the selected antimicrobial resistance genes (*ermB* and *tetO*). Hybridization was carried out at 65°C overnight, and then blot were washed three times under conditions of high stringency (20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 10% sodium dodecyl sulfate). Hybridized probes were detected by exposure to a radiographic film at −80°C overnight. Filter mating experiments were performed as previously described *(39)*. The nine *E. faecalis* isolates harboring *ermB* and *tetO* genes of the predominant multiresistant phenotype were used as donors, and *E. faecalis* JH2-2 (rif^R, fus^R; $ermB^-$) was used as the recipient strain. The PCR assay described above was performed to identify both genes in the transconjugants.

RESULTS

Bacterial identification. A total of 526 isolates from both turkeys and chicken broilers were biochemically identified to genus as *Enterococcus*. Identification to the species level using the multiplex PCR assay revealed 150 *E. faecalis* and 54 *E. faecium* isolates from turkeys and 120 *E. faecalis* and 63 *E. faecium* from chicken broilers, for a total of 387 isolates of these two *Enterococcus* species.

Multiresistance phenotypes. Percentages of antimicrobial-resistant isolates based on MICs for both *E. faecalis* ($n = 270$) and *E. faecium* ($n = 117$) from chickens and turkeys are presented in Tables 1 and 2, respectively. Although all isolates were resistant to three or more antimicrobials, all were susceptible to VAN and LIZ. Low, intermediate, and high frequencies of antimicrobial resistance were observed in the *E. faecalis* and *E. faecium* isolates tested in relation for specific antimicrobials. Most of the isolates of *E. faecalis* and *E. faecium,* respectively, were resistant to BAC (88.1 and 94%), ERY (72.6 and 80.3%), LIN (100 and 94%), QD (98.5 and 89.7%), TET (95.6 and 89.7%), and TYL (73 and 75.2%) but susceptible to CHL (0 and 0.9%). Low to intermediate frequencies of antimicrobial resistance to CIP (0.7 and 14.5%), FLA (3.7 and 41%), GEN (high level; 9.6 and 4.3%), KAN (high level; 25.2 and 17.1%), STR (high level; 46.7 and 38.5%), NIT (2.6 and 20.5%), PEN (3 and 27.4%), and SAL (7 and 12.8%) were observed in both *E. faecalis* and *E. faecium*, respectively. Multidrug resistance to LIN-QD-TET was observed in 94.4% (255) of the *E. faecalis* isolates, and multiresistance to BAC-ERY-LIN-QD-TET-TYL was found in 66.7% (78) of the *E. faecium* isolates. Among *E. faecium* isolates, a wide range of MICs of QD (4 to 32 μg/ml) was observed. Also, a high level of resistance to TET and TYL was observed, with high MICs of these antimicrobials.

Numerous multiresistant phenotypes were observed among both *E. faecalis* and *E. faecium* isolates from turkeys and chicken broilers (Table 3). *E. faecium* isolates had more multiresistant phenotypic profiles (65 different profiles) than did *E. faecalis* (42 profiles). Five common multiresistant phenotypes were observed in *E. faecalis* isolates: BAC-ERY-LIN-QD-TET-TYL (54 isolates), BAC-LIN-QD-TET (50), BAC-ERY-LIN-KAN-QD-STR-TET-TYL (36), BAC-ERY-LIN-QD-STR-TET-TYL (25), and ERY-LIN-QD-STR-TET-TYL (23). One predominant multiresistant phenotype was observed in *E. faecium* isolates: BAC-ERY-LIN-QD-TET-TYL (19 isolates). Both of the predominant multiresistance patterns in *E. faecalis* and *E. faecium* included six antimicrobials.

Multiresistance genotypes and Rep-PCR clonality. All *E. faecalis* and *E. faecium* isolates were tested for the presence of *vanA, vanB,* and *qacEd1-sul1* genes, and none of these genes were detected. For each species, PCR assay and sequencing were used to examine the genes encoding for antimicrobial resistance in the predominant multiresistant phenotype (Table 4). The *ermB* (MLS_B resistance), *bcrB,* and *tetM* genes were the most commonly observed resistance determinants. Of those isolates positive for *ermB* and *tetM* genes (65 of 73), 18 were positive for the Tn*916*/Tn*1545*-like integrase family gene. Eight isolates that were negative for the Tn*916*/Tn*1545*-like gene and were *tetM* resistant were positive for the presence of the Tn*5397*-like (*tdnX*) gene. Isolates that were positive for the Tn*916*/Tn*1545*-like integrase family gene were all *tetM* and *ermB* resistant. BAC resistance and TET resistance was not explained in 20 and 1 isolate, respectively. QD resistance was not explained in nine *E. faecium* isolates, which did not have any *vat* genes *(36)*. All other resistance genes conferring resistance to a particular antimicrobial were

TABLE 1. *Antimicrobial resistance in* E. faecalis *isolates from turkeys and broiler chickens based on MICs*[a]

Antimicrobial agent	No. of isolates at an MIC (μg/ml) of:															% resistant isolates
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	
Bacitracin							3	5	24	**49**	**189**					88.1
Chloramphenicol					1	163	105	1								0
Ciprofloxacin	2	6	94	159	7	**2**										0.7
Erythromycin			39	24	10	1	**196**									72.6
Flavomycin				243	7	4	4	2	**10**							3.7
Gentamicin											240	4	**9**	**17**		9.6
Kanamycin											193	5	4	**68**		25.2
Lincomycin							**1**	**7**	**262**							100
Linezolid			5	187	78											0
Nitrofurantoin						26	191	18	20	8	**7**					2.6
Penicillin			6	4	102	146	4	**8**								3
Salinomycin				122	56	73	**18**		**1**							7
Streptomycin													144	**7**	**119**	46.7
Quinupristin-dalfopristin					4	**9**	**179**	**48**	**30**							98.5
Tetracycline						12		**6**	**252**							95.6
Tylosin				54	16	3			**197**							73
Vancomycin			15	194	60	1										0

[a] Vertical lines indicate MIC breakpoints; bold numbers are the number of resistant isolates.

TABLE 2. *Antimicrobial resistance in* E. faecium *isolates from turkeys and broiler chickens based on MICs*[a]

Antimicrobial agent	No. of isolates at an MIC (μg/ml) of:															% resistant isolates
	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024	2,048	
Bacitracin							3	1	3	**3**	**107**					94
Chloramphenicol					6	78	32		**1**							0.9
Ciprofloxacin	1	3	13	34	49	**17**										14.5
Erythromycin			5	13	3	2	**94**									80.3
Flavomycin				28	6	21	9	5	**48**							41
Gentamicin											111	1		**5**		4.3
Kanamycin											74	21	2	**20**		17.1
Lincomycin				7			**1**	**4**	**105**							94
Linezolid			1	72	44											0
Nitrofurantoin						3	22	5	22	41	**24**					20.5
Penicillin			7	2	21	29	26	**32**								27.4
Salinomycin				26	24	52	**15**									12.8
Streptomycin													72	**20**	**25**	38.5
Quinupristin-dalfopristin				7	5	**13**	**20**	**26**	**46**							89.7
Tetracycline						11	1	**2**	**103**							89.7
Tylosin				9	16	3	1		**88**							75.2
Vancomycin			68	29	20											0

[a] Vertical lines indicate MIC breakpoints; bold numbers are the number of resistant isolates.

TABLE 3. *Main phenotypic multiresistance profiles observed among* E. faecium *and* E. faecalis *isolates from poultry*

Bacterial species	Phenotypic multiresistant profile[a]	Total no. of isolates
E. faecalis	BAC, LIN, QD, TET	50
	BAC, LIN, STR, QD, TET	8
	BAC, ERY, LIN, QD, TET, TYL	54
	ERY, LIN, STR, QD, TET, TYL	23
	BAC, ERY, LIN, STR, QD, TET, TYL	25
	BAC, ERY, GEN, KAN, LIN, QD, TET, TYL	8
	BAC, ERY, LIN, KAN, STR, QD, TET, TYL	36
	BAC, ERY, LIN, SAL, STR, QD, TET, TYL	7
	BAC, ERY, GEN, KAN, LIN, STR, QD, TET, TYL	10
E. faecium	BAC, ERY, PEN	2
	BAC, CIP, FLA, TET	2
	BAC, FLA, LIN, TET	2
	BAC, LIN, QD, TET	2
	BAC, FLA, LIN, QD	2
	BAC, LIN, STR, QD, TET	3
	BAC, ERY, LIN, QD, TET, TYL	19
	BAC, ERY, LIN, NIT, QD, TET	2
	BAC, ERY, LIN, STR, QD, TET, TYL	6
	BAC, ERY, FLA, LIN, QD, TET, TYL	7
	BAC, ERY, FLA, LIN, STR, QD, TET, TYL	3
	BAC, CIP, ERY, FLA, LIN, QD, TET, TYL	2
	BAC, ERY, FLA, LIN, PEN, QD, TET, TYL	3
	BAC, CIP, ERY, LIN, STR, QD, TET, TYL	2
	BAC, ERY, KAN, LIN, STR, QD, TET, TYL	5
	BAC, ERY, FLA, LIN, SAL, QD, TET, TYL	2
	BAC, ERY, LIN, NIT, PEN, STR, QD, TET, TYL	2
	BAC, ERY, FLA, KAN, LIN, STR, QD, TET, TYL	3
	BAC, ERY, KAN, LIN, NIT, PEN, STR, QD, TET, TYL	2

[a] BAC, bacitracin; LIN, lincomycin; QD, quinupristin-dalfopristin; TET, tetracycline; STR, streptomycin; ERY, erythromycin; TYL, tylosin; GEN, gentamicin; KAN, kanamycin; SAL, salinomycin; PEN, penicillin; CIP, ciprofloxacin; FLA, flavomycin; NIT, nitrofurantoin.

found in all isolates exhibiting the corresponding phenotype. Each isolate that had the predominant *E. faecium* multiresistant phenotype had different antimicrobial resistance genotypes, whereas 12 different antimicrobial resistance genotypes were observed for the predominant *E. faecalis* phenotype (Table 4). Of these isolates, those originating from the same pool (cecal contents of approximately 10 birds) were characterized using Rep-PCR to determine their clonality; 50% were clonal with the same antimicrobial resistance genotype. However, 45% were clonal by Rep-PCR but had different antimicrobial resistance genotypes. The remaining isolates were not clonal and had different antimicrobial resistance genes. Thus, in our study, only one representative isolate of each clone with the same antimicrobial resistance gene profile was selected.

Plasmid extraction, hybridization, and transferability. Plasmid extractions and hybridizations revealed that the *tetO* and *ermB* genes were both colocalized on a small plasmid of ca. 11 kb in nine strains of the predominant multiresistant phenotype of *E. faecalis* (Fig. 1). Only two of the nine strains were clonal, as determined by Rep-PCR. Filter mating experiments revealed that three of the strains had the capacity to transfer both genes to a recipient strain.

DISCUSSION

This study gives a better understanding of the multiple antimicrobial resistance profiles observed in *E. faecalis* and *E. faecium* isolates from the intestinal tract of healthy broiler chickens and turkeys slaughtered in Canada. For the first time, we also provide evidence of plasmid colocalization of *tetO* and *ermB* genes in *E. faecalis* isolates, indicating that antimicrobial coselection and transferability could occur via this single genetic element. *E. faecalis* was the predominant species (69.8% of isolates) recovered from both broiler chicken and turkey samples, in accordance with some reports *(29, 31)* but in contrast with others indicating *E. faecium* as the most frequent enterococcal species isolated from poultry *(34, 37)*. Both species accounted for a large proportion (74%) of the enterococcal isolates recovered from poultry in this study.

Data on phenotypic antimicrobial resistance grouping revealed that a high number of isolates of *E. faecalis* and *E. faecium* of poultry origin were resistant to different classes of antimicrobials, and the magnitude of resistance to individual antimicrobial classes was high. All *E. faecalis* and *E. faecium* isolates tested were resistant to three or more antimicrobials, and numerous multiresistance phenotypes were found in both species. The percentage of resistant isolates was generally significant, either higher or occasionally comparable to resistance previously reported for enterococci recovered from

TABLE 4. *Genotypic profiles of the predominant multiresistant phenotype (BAC-ERY-LIN-QD-TET-TYL) of* E. faecium *and* E. faecalis *isolates*

Bacterial species	Multiresistant genotypic profile	Total no. of isolates
E. faecalis	*ermB, tetM*	11
	ermB, tetO	1
	bcrB, ermB	1
	ermB, linB, tetM	1
	bcrB, ermB, tetM	4
	bcrB, ermB, tetO	2
	bcrR, bcrB, ermB, tetM	15
	bcrB, ermB, linB, tetM	1
	bcrR, bcrB, ermB, vatD, tetM	3
	bcrR, bcrB, ermB, tetM, tetO	2
	bcrR, bcrA, bcrB, ermB, vatD, tetM	1
	bcrR, bcrA, bcrB, ermB, vatD, tetO	2
E. faecium	*bcrB, ermB, tetM*	1
	ermB, msrC, tetM	1
	bcrA, bcrB, ermB, tetM	1
	bcrB, ermB, linB, tetM	1
	vatE, ermB, msrC, tetM	1
	vatE, bcrR, bcrB, ermB, tetM	1
	vatE, bcrA, bcrB, ermB, tetM	1
	bcrR, bcrA, bcrB, ermB, tetM	1
	vatE, bcrR, bcrA, bcrB, ermB, tetM	1
	vatE, bcrR, bcrB, ermB, linB, tetM	1
	vatD, bcrA, bcrB, ermB, msrC, tetM	1
	vatE, bcrB, ermB, msrC, linB, tetM	1
	bcrA, bcrB, ermB, msrC, linB, tetM	1
	bcrR, bcrA, bcrB, msrC, linB, tetM	1
	vatE, bcrR, bcrA, bcrB, ermB, linB, tetM	1
	vatE, bcrA, bcrB, ermB, msrC, linB, tetM	1
	bcrR, bcrA, bcrB, bcrD, ermB, msrC, tetM	1
	bcrR, bcrA, bcrB, bcrD, ermB, linB, tetM	1
	vatD, bcrR, bcrA, bcrB, bcrD, ermB, msrC, linB, tetM	1

poultry operations or retail meat *(2, 28, 33)*. Overall, the antimicrobial resistance we observed among our poultry enterococcal isolates is reflective of local farming practices and the commercially available antimicrobials that are favored in Canada *(5),* with some differences.

The absence of VAN resistance among enterococcal isolates from broiler chickens and turkeys is consistent with previous observations from the United States *(12)* because avoparcin has never been approved in North America. However, VAN resistance has been previously reported in poultry enterococcal isolates from European countries *(1, 2, 16),* where this type of resistance was linked to the use of avoparcin as a growth promoter.

Although a large proportion of isolates was susceptible to GEN, KAN, and STR, high-level resistance to aminoglycosides was detected in a significant percentage of isolates, in accordance with previous reports *(25, 28, 37, 40)*. In our study, prevalence of high-level resistance to STR was much higher than high-level resistance to GEN and KAN. PEN resistance was lower than resistance to STR, although PEN is reported as commonly used in Canadian poultry production both alone and in combination with STR *(5)*. GEN also is used in chickens and turkeys in North America *(25)*. However, this antimicrobial is less frequently used in Canada because of its long withdrawal period, whereas KAN is not listed for use as an antimicrobial in poultry in Canada *(5)*.

The high occurrence of QD resistance we observed among *E. faecium* isolates could be linked to the use of virginiamycin in Canadian poultry production. This observation is consistent with a previous report from the European Union *(3)*. However, it is in contrast with another report indicating that there was no correlation between use of virginiamycin and the presence of QD-resistant strains of enterococci *(24)* and with another study in which QD resistance among enterococci from animals remained low despite a long history of virginiamycin usage *(36)*. Virginiamycin and QD are both streptogramin A–B combination antimicrobials, and cross-resistance has been described *(46)*. Virginiamycin has been used extensively in agriculture as a growth promoter in many countries, but it was banned in 1998 in the European Union mainly because of concerns about resistance that might compromise streptogramin treatments in humans *(3)*. Debate continues over the contribution of virginiamycin use in animals to QD resistance in bacteria found in humans *(36)*. For *E. faecalis,* the situation appears different, because this bacterium is intrinsically resistant to streptogramin A *(27)*.

Few isolates were resistant to NIT in our study. According to one reference *(5),* this antimicrobial has not

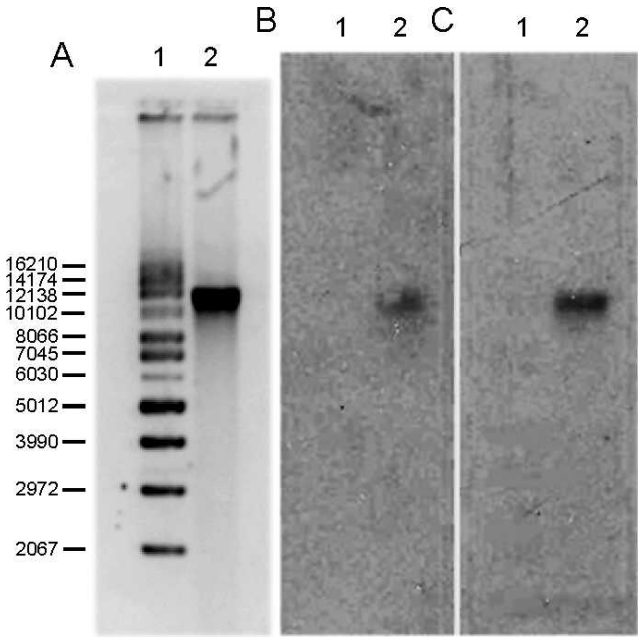

FIGURE 1. *Profiling and hybridization of undigested plasmid DNA of multiresistant* E. faecalis *strain 543. (A) Agarose gel of plasmids. (B) Southern blot of plasmids probed with* ermB. *(C) Southern blot of plasmids probed with* tetO. *Lane 1, molecular size standard (in base pairs); lane 2,* E. faecalis *strain 543 plasmids. Base pairs are indicated on the left side of the image.*

been reported to be used in poultry production in Quebec and has been banned by regulations for sale in food producing animals in Canada. Furaltadone also is not approved for veterinary use in Canada *(6)*. Therefore, NIT resistance cannot be clearly explained. Similar results were observed for NIT resistance in Portugal, which was explained by a recent massive and illicit use of furaltadone by poultry producers in this country *(22)*. Like NIT, furaltadone is a furane antimicrobial, which may explain cross-resistance to these two compounds. In Canada, furaltadone is on the veterinary new drug list, which was last updated in 2000 *(8)*.

At the molecular level, various combinations of the antimicrobial resistance genes *vatD, vatE, bcrR, bcrA, bcrB, bcrD, ermB, msrC, linB, tetM,* and *tetO* were detected in the genomes of isolates within the two predominant multiresistant phenotypes. Thus, a single multiresistant phenotype can be associated with several combinations of antimicrobial resistance genes in *E. faecalis* and *E. faecium* isolates of poultry origin. This finding is in agreement with that of a previous report indicating that poultry enterococci usually are genetically different, with little clonality *(37)*. No VAN genes were detected, and the QD *vatD* gene was observed in both *E. faecalis* and *E. faecium* isolates. The genes encoding resistance to VAN, GEN, and QD have been found in *E. faecium* isolates from humans and animals *(32)*. However, certain clones are more frequent in humans, whereas other clones predominate in various animals *(32)*. Thus, the risk of transmitting animal antimicrobial-resistant *E. faecium* isolates to humans could be of less importance, and the greater problem is the ability of human *E. faecium* isolates to acquire and transfer antimicrobial resistance genes *(32)*. In contrast, similar clones of VAN- and GEN-resistant *E. faecalis* have been recovered from both animals and humans *(32),* making the human health hazard represented by animal and meat enterococci more complex.

Rep-PCR was used to address the issue of clonality among the antimicrobial-resistant isolates of the two predominant multiresistant phenotypes originating from the same pool (cecal content of approximately 10 birds). Half of these isolates were clonal, with the same antimicrobial resistance gene profile. However, 45% of them were clonal by Rep-PCR but had different antimicrobial resistance genes. Thus, in our study, evaluation of clonality of multiresistant isolates was best performed using both Rep-PCR and molecular antimicrobial resistance analysis. Otherwise, some antimicrobial resistance genes might have been overlooked.

TET resistance was explained in all isolates except one by the *tetM* and *tetO* genes, which have been frequently reported in broilers *(2)*. TET resistance, encoded by the *tetM* gene, has been associated with conjugative transposons related to the Tn*916*/Tn*1545*-like family *(4, 23)*. Another conjugative transposon, Tn*5397*-like, has recently been found in *E. faecium* isolates from broilers *(4)*. In the present study, 28 and 17% of the *tetM*-positive enterococcal isolates carried specific Tn*916*/Tn*1545*-like genes and Tn*5397*-like transposons, respectively. This observation is consistent with a previous report *(14)*. Other researchers concluded that phenotypic acquired resistance to TET is often present in poultry enterococcal isolates that carry the *ermB* gene *(14)*.

In our study, the *ermB* (MLS_B resistance), *bcrB,* and *tetM* genes were the most commonly observed resistance genes. The *ermB* gene was colocalized with the *tetO* gene on a low-molecular-weight plasmid of ca. 11 kb in nine strains harboring these genes. To our knowledge, this is the first report of these genes on the same low-molecular-weight plasmid. Transferability was obtained in only three of those strains, suggesting a possible nonconjugative plasmid that could need mobilization by a coresident conjugative element *(19)*. TET resistance (*tetM*) and ERY resistance (*ermB)* colocalization on Tn*1545* has previously been reported in enterococci *(23)*. Tn*1545* encodes for TET (*tetM*), ERY (*ermB*), and KAN (*aph*(*3′*)-*IIIa*) resistance and has 25.3 kb *(13)*. In our study, the *tetO* and *ermB* probes both hybridized with a ca. 11-kb plasmid in *E. faecalis* isolates, whereas the *tetM* probe did not. In addition, these isolates tested negative for the Tn*916*/Tn*1545*-like integrase family gene. Thus, the *tetO* and *ermB* genes identified in this study are not colocated on the Tn*1545*-like transposon. We are sure that we identified *tetO* and not *tetM* or a hybrid of the two genes because the alignment of the sequences of the PCR product of *tetM* and *tetO* genes using nucleotide BLAST searches (NCBI, Bethesda, MD) resulted in an identity of 76% (47% query coverage). PCRs were performed on one *tetO*-positive and one *tetM*-negative strain with *tetM* primers and, inversely, with *tetO* primers on a strain with only the *tetM* gene; all these PCRs resulted in no amplifications. TETs are not frequently used in poultry operations in Canada because many microorganisms are resistant to these antimicrobials *(5)*. Thus, our results

suggest that the use of MLS_B antimicrobials could maintain TET resistance in poultry flocks via the coselection process. Results of conjugation experiments suggest that these antimicrobial resistances could be transferred to other bacteria via the ca. 11-kb plasmid.

In conclusion, we found no VAN resistance but significant high-level resistance to aminoglycosides and high occurrence of QD resistance among commensal enterococci isolated from poultry in Canada. We also determined that a single multidrug-resistant phenotype can be associated with several combinations of antimicrobial resistance genes. We also demonstrated for the first time the presence of *ermB* and *tetO* resistance genes on a small and transferable ca. 11-kb plasmid in *E. faecalis* isolates of poultry origin.

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