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Characterization of Multidrug-Resistant *Escherichia coli* Isolates from Animals Presenting at a University Veterinary Hospital▽†

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In this study, we examined molecular mechanisms associated with multidrug resistance (MDR) in a collection of *Escherichia coli* isolates recovered from hospitalized animals in Ireland. PCR and DNA sequencing were used to identify genes associated with resistance. Class 1 integrons were prevalent (94.6%) and contained gene cassettes recognized previously and implicated mainly in resistance to aminoglycosides, β-lactams, and trimethoprim (*aadA1*, *dfrA1-aadA1*, *dfrA17-aadA5*, *dfrA12-orfF-aadA2*, bla_{OXA-30}*-aadA1*, *aacC1-orf1-orf2-aadA1*, *dfr7*). Class 2 integrons (13.5%) contained the *dfrA1-sat1-aadA1* gene array. The most frequently occurring phenotypes included resistance to ampicillin (97.3%), chloramphenicol (75.4%), florfenicol (40.5%), gentamicin (54%), neomycin (43.2%), streptomycin (97.3%), sulfonamide (98.6%), and tetracycline (100%). The associated resistance determinants detected included bla_{TEM}, *cat*, *floR*, *aadB*, *aphA1*, *strA-strB*, *sul2*, and *tet*(B), respectively. The $bla_{CTX-M-2}$ gene, encoding an extended-spectrum β-lactamase (ESβL), and bla_{CMY-2}, encoding an AmpC-like enzyme, were identified in 8 and 18 isolates, respectively. The mobility of the resistance genes was demonstrated using conjugation assays with a representative selection of isolates. High-molecular-weight plasmids were found to be responsible for resistance to multiple antimicrobial compounds. The study demonstrated that animal-associated commensal *E. coli* isolates possess a diverse repertoire of transferable genetic determinants. Emergence of ESβLs and AmpC-like enzymes is particularly significant. To our knowledge, the $bla_{CTX-M-2}$ gene has not previously been reported in Ireland.

Antimicrobial agents are extensively used in animal therapy, for prophylaxis and metaphylaxis, and in some geographical regions for growth promotion. Many of these drugs belong to the same families of antimicrobial compounds that are used for treating humans (76). Surveillance studies conducted in different countries generally report an increase in the level of resistance in *Escherichia coli* isolates to major classes of antibiotics used for the treatment of livestock and companion animals (28, 34). It has been suggested that an important factor in the emergence and dissemination of resistance is the selective pressure exerted following antibiotic exposure.

Drug-resistant commensal *Escherichia coli* isolates may constitute a significant reservoir of antibiotic resistance determinants which can spread to those bacteria pathogenic for animals and/or humans (77). For this reason, the reported emergence of resistance to new drugs such as extended-spectrum β-lactams and fluoroquinolones (FQs) is of particular concern to both animal and public health alike. The potential for transmission of *E. coli* clones between different animal hosts and humans has been documented previously (26). In addition, transmission of genetic determinants of resistance *in vitro* and *in vivo* has been described in several studies (13, 37, 49). Multidrug-resistant (MDR) *E. coli* strains are often isolated from animals in veterinary hospitals and are frequently associated with opportunistic infections (59). However, currently, detailed knowledge of the resistance mechanisms in bacteria cultured from hospitalized animals is limited.

The dissemination of resistance markers can be attributed to a number of independent genetic events collectively known as horizontal gene transfer (HGT). Most of the genes conferring antibiotic resistance are not specific to one bacterial host. HGT has been attributed to mobile and mobilizable genetic elements such as plasmids, transposons, and integrons since resistance determinants frequently map within these structures. Furthermore, many of the resistance markers persist despite the withdrawal of certain antibiotics from use in therapy (2, 58). The physical linkage of genes, in some cases at least, may explain these epidemiological data (65). Transfer of resistance plasmids between bacteria of veterinary importance, including *E. coli*, has been demonstrated *in vitro* and *in vivo* (14, 49).

Integrons are genetic structures involved in the transmission and expression of the resistance genes located within gene cassettes. Several classes of integrons are recognized on the basis of the sequence of specific recombinases known as integrase, of which class 1 is the most clinically relevant. Their 5′ conserved segment (5′-CS) contains *intI1*, a gene encoding the integrase along with a promoter region (P_c) required for the expression of the gene cassette. The 3′-CS typically contains a defective quaternary ammonium compound resistance gene denoted *qacEΔ1*, followed by s*ul1*, conferring sulfonamide re-

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sistance. Class 1 integrons have emerged and continue to evolve as a result of site-specific recombination events catalyzed by the *intI1*-encoding enzyme between the *attI1* site of the 5′-CS and the 59-base element located toward the distal end of the gene cassette (21, 52, 55). As integrons are frequently present in MDR bacteria and are associated with conjugative broad-host-range plasmids and transposons, their role in the evolution and dissemination of resistance among different bacterial species and genera is widely recognized (8, 9, 52).

While antibiotic-resistant zoonotic food-borne pathogens constitute an obvious threat to public health, the problem of resistance in other bacteria colonizing animals cannot be ignored. Data on the genetic mechanisms of antibiotic resistance in both food-producing and companion animals in Ireland are limited. This study was undertaken to characterize MDR in a diverse collection of animal *E. coli* isolates at the molecular level and evaluate the potential risk of resistance determinant(s) transmission.

MATERIALS AND METHODS

Selection of bacterial isolates for the study and resistance profiling. A collection of 74 *E. coli* isolates resistant to 3 or more antimicrobial compounds of different classes was cultured from samples from selected animals presenting at the University Veterinary Hospital in Dublin, Ireland, between February and December 2007. These were consecutive MDR clinical isolates processed for routine laboratory testing. Forty-four equine isolates, 17 bovine isolates, 9 porcine isolates, 3 canine isolates, and 1 ovine isolate of *E. coli* were recovered from a variety of samples, predominantly fecal. Prior to testing, isolates were streaked for purity on MacConkey agar (Oxoid, Basingstoke, England), subcultured to tryptic soy agar (Oxoid), and stored at −80°C on cryoprotectant beads (Technical Service Consultants Ltd., Lancashire, England). Susceptibility testing by disc diffusion was performed, and results were interpreted as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (11). Susceptibility testing included a panel of 19 different antimicrobial agents: amikacin (Ak), 30 μg; amoxicillin-clavulanic acid (Amc), 20/10 μg; ampicillin (Amp), 10 μg; cefpirome (Cfp), 30 μg; cefpodoxime (Cpd), 10 μg; ceftiofur (Cfr), 30 μg; cephalothin (Cpl), 30 μg; chloramphenicol (C), 30 μg; ciprofloxacin (Cip), 5 μg; colistin (Ct), 25 μg; florfenicol (Ffc), 30 μg; furazolidone (Fr), 15 μg; gentamicin (Gm), 10 μg; neomycin (Neo), 30 μg; nalidixic acid (Na), 30 μg; streptomycin (S), 10 μg; sulfonamide compound (Su), 300 μg; tetracycline (Te), 30 μg; and trimethoprim (Tmp), 5 μg. The discs were purchased from Oxoid, and *E. coli* ATCC 25922 was included as a quality control strain.

Phylogenetic grouping. Phylogenetic groups were determined for each *E. coli* isolate using an established multiplex PCR targeting *chuA*, *yja*, and TSPE4.7 according to the protocol of Clermont et al. (10). The method was previously developed to classify *E. coli* into 4 phylogenetic groups designated A, B1, B2, and D. Target amplification was performed using the original primer concentrations and cycling conditions. Amplicons generated were separated by conventional 1.7% (wt/vol) agarose gel electrophoresis, stained with 0.1 μg/ml ethidium bromide (Sigma-Aldrich, Ireland) in 0.5× Tris-EDTA-boric acid buffer, and subsequently assigned to one of the lineages (A, B1, B2, or D) using the criteria outlined previously (10).

Detection of antibiotic resistance genes and class 1 and class 2 integrons. Total genomic DNA was extracted using a Promega Wizard genomic DNA purification kit (Madison, WI) following the manufacturer's protocol. The DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer (Thermoscientific, Wilmington, DE). Detection of antibiotic resistance markers and integron-associated genes was performed using the primers listed in Table 1. The following resistance determinants were investigated by PCR: *aac(3)-IV*, *aadA*, *aadB*, *ampC*, *aphA1*, *aphA2*, bla_{CARB}, bla_{CMY-2}, bla_{CTX-M}, bla_{OXA}, bla_{SHV}, bla_{TEM}, *cat1*, *cmlA*, *floR*, *strA-strB*, *sul1*, *sul2*, *sul3*, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G). Previously published PCR methods were employed to determine the presence of conserved integron-associated genes, including *intI1* and *intI2* (coding for integrases of classes 1 and 2, respectively), *qacE*Δ*1*, *sul1*, and the variable regions of class 1 and 2 integrons (Table 1).

All PCRs were performed in a final volume of 25 μl consisting of 2.5 μl 10× PCR buffer (New England BioLabs, Ipswich, MA), 25 pmol of each primer (MWG-Biotech AG, Ebersberg, Germany), deoxynucleoside triphosphates at a concentration of 200 μM (Promega), 1 U *Taq* DNA polymerase (New England BioLabs), and 50 ng of genomic DNA.

Sequencing of integron gene cassettes and β-lactam resistance genes. Amplicons produced using degenerate primer pairs targeting variable regions of class 1 and class 2 integrons and PCR-amplified bla_{CTX} and bla_{TEM} genes of isolates displaying cefpirome resistance were purified using a Promega Wizard PCR and a gel purification system and sequenced commercially by Qiagen Sequencing Services (Qiagen, Hilden, Germany). Sequences were initially assembled using DNAStar software (DNAStar Inc., Madison, WI), and similarity searches were carried out using the BLAST program, available at the NCBI BLAST homepage (http://www.ncbi.nlm.nih.gov/BLAST/).

Plasmid profiling. Plasmid DNA was purified using a QuickGene plasmid kit S (Fuji, Tokyo, Japan) according to the manufacturer's instructions, followed by electrophoresis in a 0.7% agarose gel (SeaKem LE agarose; Lonza Wokingham, Ltd., United Kingdom) as described above. Two *E. coli* strains, V517 and 39R861, containing multiple reference plasmids were used as controls and size markers (41).

Conjugation experiments and verification. The ability to transfer antimicrobial resistance determinants was tested by broth mating experiments for 8 isolates, all of which were selected on the basis of their heterogeneous plasmid profiles and resistance genes. Rifampin-resistant, lactose-negative strain *E. coli* 26R793 served as a recipient in the conjugation assays. In brief, equal volumes of overnight cultures of the donor and recipient strains grown in Luria-Bertani (LB) broth (Difco Laboratories, Becton Dickinson, Sparks, MD) were mixed and incubated at 37°C for 18 h. The transconjugants were selected on MacConkey agar (Oxoid) supplemented with 100 μg/ml rifampin (Sigma-Aldrich) along with either 50 μg/ml ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml nalidixic acid, 50 μg/ml streptomycin, 30 μg/ml tetracycline, or 50 μg/ml trimethoprim (Sigma-Aldrich, Ireland). Presumptive transconjugants (3 colonies from each antibiotic selection) were subjected to further investigation. Susceptibility tests were performed to confirm transfer as described previously, followed by PCR to examine which resistance genes and integrons had been transferred. A selection of the transconjugants exhibiting different resistance patterns was subjected to plasmid extraction.

RESULTS

Phylogenetic classification. Isolates belonging to phylogenetic group A were found to be the most abundant in the collection ($n = 38$; 51%). Twenty-eight percent ($n = 21$) of the isolates were grouped in the B1 lineage. No isolate was identified in B2, the phylogenetic lineage associated with virulent extraintestinal strains. Twenty percent of the isolates ($n = 15$) were assigned to group D, which is associated with pathogenic bacteria, although less frequently than group B2 (10).

Resistance patterns. All isolates studied were resistant to several antimicrobial agents, ranging from 5 to 15 of the antimicrobial compounds tested (see Table S1 in the supplemental material). Resistance to tetracycline ($n = 74$; 100%), trimethoprim ($n = 74$; 100%), sulfonamides ($n = 73$; 98.6%), ampicillin ($n = 72$; 97.3%), and streptomycin ($n = 72$; 97.3%) was the most common, followed by cephalothin ($n = 54$; 73%), chloramphenicol ($n = 51$; 75.4%), the amoxicillin-clavulanic acid combination ($n = 42$; 56.8%), cefpodoxime ($n = 41$; 55.4%), nalidixic acid ($n = 41$; 55.4%), gentamicin ($n = 40$; 54%), and ciprofloxacin ($n = 38$; 51.4%). Thirty-two isolates (43.2%) displayed resistance to neomycin, 30 (40.5%) to florfenicol, 30 (40.5%) to ceftiofur, 9 (12.2%) to cefpirome, and 4 (5.4%) to furazolidone. No amikacin or colistin resistance was observed.

Occurrence of resistance determinants. Genotyping data corresponded with the resistance phenotypes determined, and in most cases of resistance, previously recognized marker genes were identified.

Resistance to streptomycin was mediated mainly by the *strA-strB* gene pair, present in 60 isolates (81%), and *aadA*, identi-

TABLE 1. PCR primer characteristics

Target gene	Primer direction[a]	Nucleotide sequence (5′–3′)	Annealing temp (°C)	Amplicon size (bp)	Reference
intI1	F	CAG TGG ACA TAA GCC TGT TC	59	160	Koeleman et al. (31)
	R	CCC GAG GCA TAG ACT GTA			
Class 1 gene cassette	F	GGC ATC CAA GCA GCA AGC	55	Variable	Lévesque et al. (36)
	R	AAG CAG ACT TGA CCT GAT			
qacEΔ1	F	ATC GCA ATA GTT GGC GAA GT	53	250	Sandvang et al. (60)
	R	GAA GCT TTT GCC CAT GAA GC			
sul1	F	CGG CGT GGG CTA CCT GAA CG	66	433	Kerrn et al. (29)
	R	GCC GAT CGC GTG AAG TTC CG			
intI2	F	CAC GGA TAT GCG ACA AAA AGG T	54	788	Mazel et al. (45)
	R	GTA GCA AAC GAG TGA CGA AAT G			
Class 2 gene cassette	F	CGG GAT CCC GGA CGG CAT GCA CGA TTT GTA	62	Variable	White et al. (75)
	R	GAT GCC ATC GCA AGT ACG AG			
aadA	F	GTG GAT GGC GGC CTG AAG CC	60	525	Madsen et al. (42)
	R	AAT GCC CAG TCG GCA GCG			
strA-strB	F	ATG GTG GAC CCT AAA ACT CT	60	893	Tamang et al. (66)
	R	CGT CTA GGA TCG AGA CAA AG			
aac(3)-IV	F	TGC TGG TCC ACA GCT CCT TC	60	653	Boerlin et al. (7)
	R	CGG ATG CAG GAA GAT CAA			
aphA1	F	ATG GGC TCG CGA TAA TGT C	53	600	Maynard et al. (44)
	R	CTC ACC GAG GCA GTT CCA T			
aphA2	F	GAT TGA ACA AGA TGG ATT GC	53	347	Travis et al. (68)
	R	CCA TGA TGG ATA CTT TCT CG			
aadB	F	GAG GAG TTG GAC TAT GGA TT	53	208	Travis et al. (68)
	R	CTT CAT CGG CAT AGT AAA A			
ampC	F	CCC CGC TTA TAG AGC AAC AA	53	634	Féria et al. (18)
	R	TCA ATG GTC GAC TTC ACA CC			
bla_{CARB}	F	CAA GTA CTT TYA AAA CAA TAG C	50	534	Henriques et al. (24)
	R	GCTGTAATACTCCKAGCAC			
bla_{CMY-2}	F	AAC ACA CTG ATT GCG TCT GAC	60	1,226	Pérez-Pérez and Hanson (53)
	R	CTG GGC CTC ATC GTC AGT TA			
bla_{CTX}	F	CGA TGT GCA GTA CCA GTA A	60	585	Batchelor et al. (4)
	R	TTA GTG ACC AGA ATC AGC GG			
$bla_{CTX-SEQ}$[b]	F	TTA ATG ATG ACT CAG AGC ATT C	52	902	Villegas et al. (72)
	R	GAT ACC TCG CTC CAT TTA TTG			
bla_{OXA}	F	TAT CTA CAG CAG CGC CAG TG	53	199	Féria et al. (18)
	R	CGC ATC AAA TGC CAT AAG TG			
bla_{SHV}	F	TCA GCG AAA AAC ACC TTG	53	475	M'Zali et al. (48)
	R	TCC CGC AGA TAA ATC ACCA			
bla_{TEM}	F	TAC GAT ACG GGA GGG CTT AC	53	716	Belaaouaj et al. (5)
	R	TTC CTG TTT TTG CTC ACC CA			
$bla_{TEM-SEQ}$[b]	F	GGG GAG CTC ATA AAA TTC TTG AAG AC	59	1,100	Spanu et al. (64)
	R	GGG GGA TCC TTA CCA ATG CTT AAT CA			
cat	F	AGT TGC TCA ATG TAC CTA TAA CC	55	547	Van et al. (70)
	R	TTG TAA TTC ATT AAG CAT TCT GCC			
cmlA	F	CCG CCA CGG TGT TGT TGT TAT C	59	698	Keyes et al. (30)
	R	CAC CTT GCC TGC CCA TCA TTA G			
floR	F	TAT CTC CCT GTC GTT CCA G	53	399	Keyes et al. (30)
	R	AGA ACT CGC CGA TCA ATG			
sul2	F	CGG CAT CGT CAA CAT AAC CT	66	721	Lanz et al. (34)
	R	TGT GCG GAT GAA GTC AGC TC			
sul3	F	CAA CGG AAG TGG GCG TTG TGG A	66	244	Kozak et al. (32)
	R	GCT GCA CCA ATT CGC TGA ACG			
tet(A)	F	GCT ACA TCC TGC TTG CCT TC	55	210	Ng et al. (51)
	R	CAT AGA TCG CCG TGA AGA GG			
tet(B)	F	TTG GTT AGG GGC AAG TTT TG	55	659	Ng et al. (51)
	R	GTA ATG GGC CAA TAA CAC CG			
tet(C)	F	CTT GAG AGC CTT CAA CCC AG	55	418	Ng et al. (51)
	R	ATG GTC GTC ATC TAC CTG CC			
tet(D)	F	AAA CCA TTA CGG CAT TCT GC	55	787	Ng et al. (51)
	R	GAC CGG ATA CAC CAT CCA TC			
tet(E)	F	AAA CCA CAT CCT CCA TAC GC	55	278	Ng et al. (51)
	R	AAA TAG GCC ACA ACC GTC AG			
tet(G)	F	GCT CGG TGG TAT CTC TGC TC	55	468	Ng et al. (51)
	R	AGC AAC AGA ATC GGG AAC AC			

[a] F, forward; R, reverse.
[b] Primers $bla_{CTX-SEQ}$ and $bla_{TEM-SEQ}$ were used to PCR amplify bla_{CTX} and bla_{TEM} genes for sequencing, respectively.

fied in 57 isolates (77%). Two isolates phenotypically susceptible to streptomycin (isolates 18 and 19; see Table S1 in the supplemental material) both carried *strA-strB*, and another isolate, isolate 18, also possessed *aadA*. All of the isolates displaying neomycin resistance ($n = 31$) harbored the *aphA1* determinant. The *aphA1* marker was also present in 9 neomycin-susceptible isolates (a total of 40 *aphA1*-positive isolates in the collection; 54%). Nineteen isolates (25.7%) were positive for *aadB*, encoding gentamicin resistance. Seventeen of these were phenotypically resistant, while 2 were susceptible to this antibiotic (isolates 5B and 33). The underlying mechanisms of gentamicin resistance were not identified in a further 23 *aadB*-negative isolates, all of which showed high-level resistance to this compound. Simultaneous carriage of multiple aminoglycoside resistance determinants was common.

Genes encoding various exogenous β-lactamases were identified in all but 1 (isolate 5B) of the 72 isolates that were defined as being resistant to at least one drug within this class. The bla_{TEM} gene was the most prevalent ($n = 66$; 89.2%), followed by $bla_{\mathrm{CMY\text{-}2}}$ ($n = 18$; 24.3%), bla_{CARB} ($n = 11$; 14.9%), bla_{CTX} ($n = 8$; 10.8%), bla_{SHV} ($n = 5$; 6.8%), and bla_{OXA} ($n = 1$; 1.35%). Twenty and 9 isolates simultaneously contained 2 and 3 β-lactam-resistance-associated markers, respectively. The β-lactam resistance phenotypes of the isolates that carried bla_{TEM} (the only detectable β-lactamase gene) differed greatly and were as follows: ampicillin resistance only ($n = 9$); ampicillin and amoxicillin-clavulanate resistance ($n = 7$); ampicillin and cephalothin resistance ($n = 7$); ampicillin, amoxicillin-clavulanate, and cephalothin resistance ($n = 5$); ampicillin, cefpodoxime, and cephalothin resistance ($n = 1$); ampicillin, amoxicillin-clavulanate, cefpodoxime, and cephalothin resistance ($n = 7$); and amoxicillin-clavulanate, ampicillin, amoxicillin-clavulanate, cefpirome, cefpodoxime, ceftiofur, and cephalothin resistance ($n = 1$). In contrast, bla_{CTX}-positive isolates, all of which also carried bla_{TEM}, had identical resistance patterns and were resistant to the full panel of β-lactam compounds tested, except for the amoxicillin-clavulanate combination. Of the 18 $bla_{\mathrm{CMY\text{-}2}}$-positive isolates, 14 also carried bla_{TEM} and 9 also carried bla_{CARB}. All were resistant to a range of β-lactams, including ampicillin, amoxicillin-clavulanate, cefpodoxime, and cephalothin, and 15 displayed additional resistance to ceftiofur. The bla_{SHV} genes were always accompanied by bla_{TEM}, and 4 of 5 isolates that were positive for both of these genes were resistant to ampicillin, cefpodoxime, ceftiofur, and cephalothin, while the remaining isolate displayed resistance to ampicillin and amoxicillin-clavulanate. Isolate 29, which was positive for bla_{OXA}, was also determined to be resistant to ampicillin and amoxicillin-clavulanate. β-Lactam-susceptible isolates did not possess any of these markers, as determined by PCR, except for the endogenous *ampC*.

Mechanisms of chloramphenicol resistance were identified for 47 of the 51 resistant isolates. The *cat* gene, encoding chloramphenicol acetyltransferase, was identified in 29 strains (21.5%), whereas the prevalence of *cmlA* conferring nonenzymatic resistance was significantly lower ($n = 12$; 8.9%). The chloramphenicol-florfenicol transporter *floR* was detected in 28 (20.7%) isolates, all of which were coresistant to florfenicol, as expected. This gene was frequently detected along with *cat* ($n = 10$), *cmlA* ($n = 10$), or both genes ($n = 1$). In addition, 2 isolates (isolates 21 and CL59) classified florfenicol resistant did not carry *floR* but were positive by PCR for the *cat* gene. No associated resistance genes were detected in the 23 isolates susceptible to phenicols.

TABLE 2. Integron-associated gene cassettes identified in MDR *E. coli* isolates recovered from animals presenting at the University Veterinary Hospital

Integron class	Approximate size (kb)	Gene cassette array
1	0.8	*dfr7*
1	1.0	*aadA1*
1	1.3	*aadA1*
1	1.5	*dfrA1-aadA1*
1	1.7	*dfrA17-aadA5*
1	2.0	*dfrA12-orfF-aadA2*
1	2.0	$bla_{\mathrm{OXA\text{-}30}}$*-aadA1*
1	2.7	*aacC1-orf1-orf2- aadA1*
2	2.6	*dfrA1-sat1-aadA1*

Resistance to sulfonamides was mediated by the *sul2* gene in 66 (89.2%) and the *sul1* gene in 56 (75.7%) of the 73 sulfonamide-resistant isolates. Forty-five isolates possessed both *sul1* and *sul2*. A single isolate (denoted isolate 17; see Table S1 in the supplemental material) possessed the *sul3* marker. A single sulfonamide-susceptible isolate did not carry any of the known *sul* genes.

Tetracycline resistance, observed in all isolates of this collection, was mediated predominantly by *tet*(B), identified in 37 (50%) isolates, or *tet*(A), identified in 31 isolates (41.9%), while 3 isolates (4%) were found to possess *tet*(D). Six of these (8.1%) possessed both *tet*(A) and *tet*(B). Tetracycline resistance genes were not identified in the further 9 isolates classified to be resistant to this antibiotic.

Sequence analysis of selected β-lactamase genes. Nucleotide sequence analysis revealed that all 8 cefpirome-resistant isolates that were positive for bla_{TEM} and bla_{CTX} carried identical variants of these genes and included $bla_{\mathrm{TEM\text{-}1}}$, encoding a classical narrow-spectrum β-lactamase conferring ampicillin resistance, and $bla_{\mathrm{CTX\text{-}M\text{-}2}}$, conferring the extended-spectrum-β-lactam resistance phenotype.

Integrons and gene cassettes. Seventy isolates (94.6%) were determined to possess the class 1 integrase gene (*intI1*), and most of these ($n = 56$; 76%) also carried both the *qacEΔ1* and *sul1* genes, located within the 3′-CS. PCR amplification with consensus primers targeting the regions flanking the gene cassettes yielded 11 different gene cassettes. Integrase I (*intI1*)-positive isolates possessed none ($n = 9$; 12.2%), 1 ($n = 53$; 71.6%), 2 ($n = 4$; 5.4%), or 3 ($n = 4$; 5.4%) gene cassettes. The following amplicons and their corresponding sizes were identified: 800 bp ($n = 1$; 1.4%), 1 kb ($n = 17$; 23%), 1.3 kb ($n = 4$; 5.4%), 1.5 kb ($n = 45$; 60.8%), 1.7 kb ($n = 5$; 6.8%), 2 kb ($n = 5$; 6.8%), and 2.7 kb ($n = 6$; 8.1%). A representative of each of these gene cassettes was subjected to DNA sequencing. A summary of the sequencing data is provided in Table 2, and a genetic map is shown in Fig. 1.

The 800-bp amplicon contained a single open reading frame (ORF) that was 99% similar to a previously reported *dfr7* gene cassette (GenBank accession no. X58425). The *aadA1* gene, encoding an aminoglycoside-modifying enzyme, was identified within the variable regions of both the 1- and 1.3-kb cassettes (99% similarity to a sequence from GenBank with accession

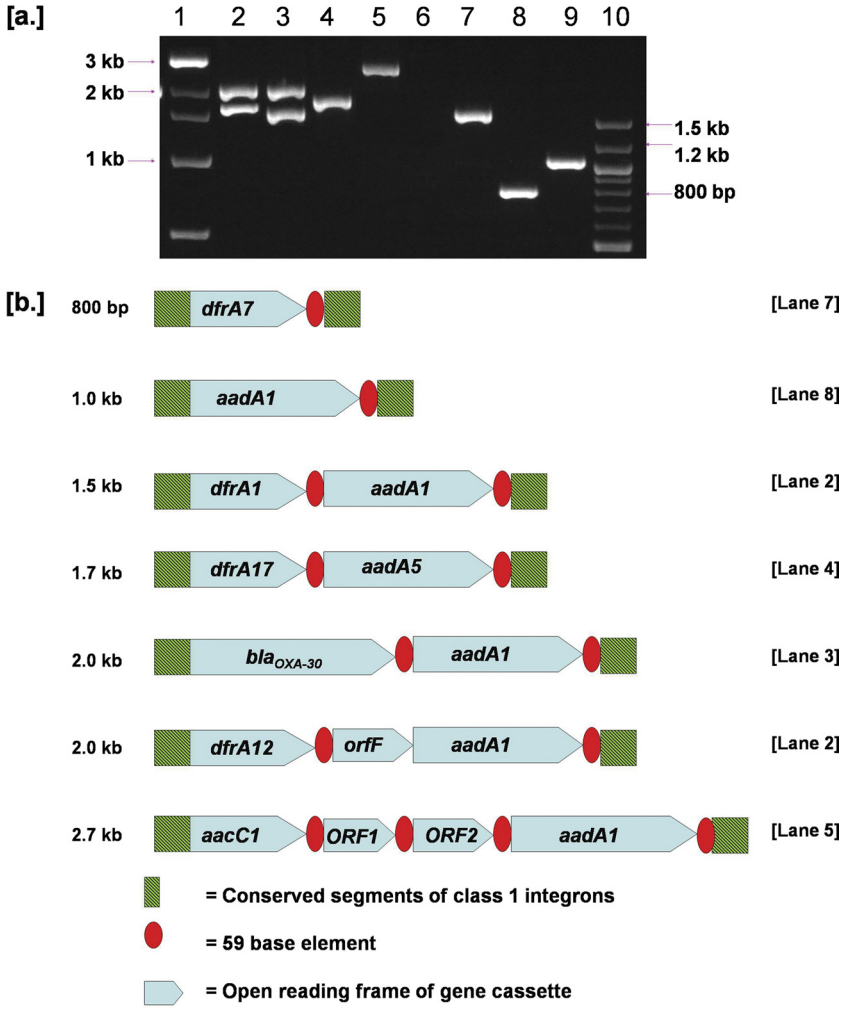

FIG. 1. (a) Representation of the sequenced gene cassettes amplified with primers targeting conserved segments of the class 1 integrons after electrophoretical separation in 1.7% agarose gel. Lane 1, 1-kb DNA size marker (New England BioLabs); lane 2, *E. coli* 18; lane 3, *E. coli* 29; lane 4, *E. coli* 28; lane 5, *E. coli* 21; lane 6, empty; lane 7, *E. coli* 16; lane 8, *E. coli* 2; lane 9, *E. coli* CL45; lane 10, 100-bp DNA size marker. (b) Schematic representation of the organization of the sequenced gene cassettes of the class 1 integrons. Arrowheads represent the direction of the transcription. Integron-associated conserved features are indicated.

no. EF078697). Variable regions of 1.5 kb contained an array of the *dfrA1* and *aadA1* genes, associated with trimethoprim and streptomycin resistance, respectively (99% similarity with the sequence with GenBank accession no. GQ293501). The variable region of 1.7 kb contained a *dfrA17* marker conferring resistance to trimethoprim and the aminoglycoside acetyltransferase *aadA5* gene conferring resistance to streptomycin-spectinomycin and exhibited 100% similarity to the sequence with GenBank accession no. GU055937. Sequence analysis of the 2-kb amplicon revealed that it contained a *dfrA12-orfF-aadA2* gene arrangement with 99% similarity to the sequence with GenBank accession no. HM043573. Another amplicon of approximately 2 kb from isolate 29 (see Table S1 in the supplemental material) was positive for bla_{OXA} and found to contain this gene located within the gene cassette with the classical head-to-tail $bla_{\mathrm{OXA\text{-}30}}$-*aadA1* arrangement. These genes were identical to a sequence previously deposited in GenBank (accession no. F543148). The *aacC1* gene (encoding a 3-*N*-aminoglycoside acetyltransferase, involved in gentamicin resistance), *orf1*, *orf2*, and the *aadA1* gene array were identified within the 2.7-kb variable region (99% similarity to the sequence with GenBank accession no. AF550679).

Ten isolates (13.5%) possessed a class 2 integrase gene along with a 2.6-kb variable region containing a *dfrA1-sat1-aadA1* gene cassette which exhibited 100% nucleotide sequence similarity to a sequence deposited in the GenBank database under accession no. AB188272.

Analysis of plasmid content. Overall, 35 diverse plasmid profiles were observed in the strain collection (data not shown). Nineteen isolates carried single plasmids and 17 possessed 2 plasmids, while 13 and 5 isolates carried 3 and 4 plasmids, respectively. No plasmids were detected in 20 (27%) of the 74 isolates. Large plasmids with sizes equal to or greater than 50 kb were observed in 40 isolates (54%).

Conjugal transfer of resistance markers. Eight isolates were selected and tested for their ability to transfer one or more of the resistance markers to a susceptible *E. coli* recipient (Table 3). Resistance to between 1 and 5 antimicrobial classes was

TABLE 3. Summary of phenotypic and genotypic characterization of transconjugants obtained in broth mating assays with MDR *E. coli* isolates from animals presenting at the University Veterinary Hospital[a]

Strain no./ origin	Resistance profile of donor	Resistance transferred to *E. coli* recipient/resistance gene(s) identified in transconjugants[d]	Plasmid(s) acquired (kb)
11/equine	AmcAmpCCfrCipCpdCplGmNaSSuTeTmp	AmcAmpCfrCpdCpl/bla_{CMY-2}	~100
		AmcAmpCfrCpdCplGmS/*intI1*–1-kb GC[b](1)–*qacEΔ1–aadA–*bla_{CMY-2}	~100
		AmcAmpCfrCpdCplSSuTmp/*intI1*–1.5-kb GC(1)–*qacEΔ1–sul1–*bla_{CMY-2}–bla_{TEM}–*strA-strB–sul2*	2×[c] ~100
15/equine	AmcAmpCCfrCipCpdCplFfcNNaSSuTeTmp	AmpGmNS/*aphA1–*bla_{TEM}–*strA-strB*	~100
		AmpGmNSSuTeTmp/*intI1*–1.5-kb GC(1)–*qacEΔ1–sul1–aadA–aphA1–*bla_{TEM}–*strA-strB–sul2–tet*(A)	~7
31/canine	AmcAmpCCipFfcNaSSuTeTmp	CFfcSSuTe/*floR–strA-strB--sul2–tet*(B)	~150
32/canine	AmcAmpCCipFfcNaSSuTeTmp	AmcAmp/bla_{TEM}	~100
		CFfcSSuTe/*floR–strA-strB–sul2–tet*(B)	2× ~100
CL42/equine	AmpCCfpCfrCpdCplGmNSSuTeTmp	AmpCCfpCfrCpdCplGmNSSuTeTmp/*intI1*–1.5-kb GC(1)–*qacEΔ1–sul1–aadA–aphA1–*$bla_{CTX-M-2}$–bla_{TEM}–*cat–strA-strB–sul2–tet*(B)	~150
		AmpCCfpCfrCpdCplGmNSuTeTmp/*intI1*–1.5-kb GC(1)–*qacEΔ1–sul1–aadA–aphA1–*$bla_{CTX-M-2}$–*cat–tet*(B)	
CL62/equine	AmcAmpCCfrCpdCplFfcGmNSSuTeTmp	AmpNSSuTe/*aphA1–*bla_{TEM}–*strA-strB–sul2–tet*(A)	~150

[a] Two independent mating-out experiments were performed. Resistance transfer was not observed with *E. coli* isolates 6 and 29.
[b] GC, gene cassette.
[c] 2×, the presence of two plasmids of equal approximate size.
[d] (1) indicates the presence of a class 1 integron type.

transferred, and multiresistance transfer occurred frequently. One isolate (*E. coli* CL42; see Table S1 in the supplemental material) could transfer its entire resistance repertoire (resistance to 12 antimicrobial compounds). Relevant genes matching the phenotypic resistance profiles were also identified (Table 3). Plasmid profiling revealed that transconjugants principally acquired high-molecular-weight plasmids of approximately 100 kb or larger (Fig. 2).

DISCUSSION

In veterinary hospitals, the ongoing usage of antimicrobial compounds for treatment of animals increases the selective pressure for emergence of MDR organisms and dissemination of resistance (16, 50). Antimicrobial agents frequently used in referral veterinary hospitals include broad-spectrum-activity drugs, such as β-lactams and new cephalosporins, as well as fluoroquinolones. Thus, hospitalized animals may constitute an important reservoir of antimicrobial resistance (20).

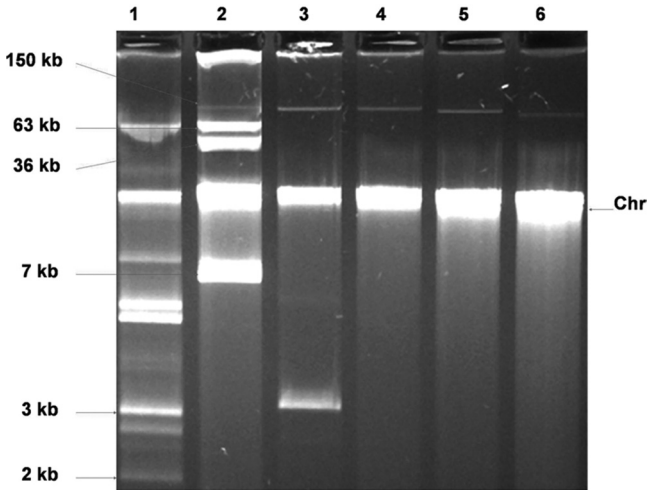

FIG. 2. Agarose gel image showing plasmid profiles of *E. coli* 31 and its transconjugants. Chr, chromosomal DNA. Lane 1, *E. coli* 39R861; lane 2, *E. coli* V517; lane 3, *E. coli* 31 (AmcAmpCCipFfc NaSSuTeTmp resistance profile); lanes 4, 5, and 6, *E. coli* 31-derived transconjugants selected on chloramphenicol, streptomycin, and tetracycline, respectively, all with the CFfcSSuTe resistance profile.

Of the 74 *E. coli* isolates investigated in this study, the majority (80%) belonged to phylogroups A and B1 and are therefore classified commensal bacteria, but a number of strains, within group D (20%), were classified potentially pathogenic. Bacteria of classes A and B1 are rarely reported to possess virulence genes (10). However, although typing of isolates from humans showed a good correlation of phylogenetic group with pathogenicity (10), animal-associated extraintestinal pathogenic *E. coli* (ExPEC) can be phylogenetically distinct. Thus, caution should be exercised when defining such bacteria as commensals (19, 22). Furthermore, commensal *E. coli* strains can cause extraintestinal disease when predisposing factors for infection are present (57). Therefore, although most of the isolates investigated in this study may be phylogenetically classified commensal organisms, they are not representative of commensal strains from healthy animals in the community.

The presence of class 1 integrons, associated with resistance to trimethoprim, streptomycin, and sulfonamide, is in agreement with previous investigations. The variable gene cassette regions characterized in this study were previously reported and are widely disseminated among the *Enterobacteriaceae* (28, 40). In addition, identical class 2 integrons, associated with Tn*7* and Tn*7*-like transposable elements, have been identified previously (3, 40, 73). The absence of the 3′ conserved structure (containing *qacEΔ1-sul1*) in several isolates has been reported on occasions by others, and such genetic elements are sometimes associated with the *sul3* gene (63).

Molecular investigations of the underlying aminoglycoside resistance mechanisms revealed that the *strA-strB* gene pair was the most prevalent (81%) among the determinants identified, followed by *aadA* (77%) and the less frequent *aphA1*

(54%) and *aadB* (25.7%) markers. This observation is consistent with earlier reports showing that these genes are common in isolates resistant to streptomycin and/or other aminoglycoside drugs (65). The fact that a*phA1* was present in 9 isolates phenotypically susceptible to neomycin and, likewise, the presence of *strA-strB* and/or *aadA* in streptomycin-susceptible isolates could be explained by the existence of defective genes or reduced expression of these markers, required to confer the phenotype.

The most commonly detected β-lactamase gene in this study was bla_{TEM}, occurring in 66 of 74 strains. The prevalence of bla_{TEM} is in good agreement with previous reports that found this marker to be widely disseminated among the *Enterobacteriaceae* from veterinary sources (38). The diversity of the β-lactam resistance phenotype among bla_{TEM}-positive isolates from the current strain collection may be due to the presence of altered gene variants in some isolates or the overexpression of endogenous, chromosomally encoded AmpC enzyme (27). TEM enzymes, including ESβLs, are typically inhibited by clavulanic acid. Nonetheless, we demonstrated the transfer of ampicillin and amoxicillin-clavulanate resistance from a canine isolate (*E. coli* isolate 32) by conjugation (Table 3) that was directly linked with the presence of bla_{TEM}, indicating that an inhibitor-resistant enzyme variant was present. Inhibitor-resistant TEM enzymes (IRT) have been reported worldwide (5, 43).

The *shv* determinant was detected in a smaller number of isolates: a canine isolate (denoted isolate 31 in Table S1 in the supplemental material) was resistant to ampicillin and amoxicillin-clavulanate, along with 4 equine fecal isolates (denoted isolates CL50, CL61, CL64, and CL78). It is possible that variants of these enzymes in this isolate collection may have arisen following different levels of selective pressure. Moreover, due to the concomitant presence of bla_{TEM} genes, it is difficult to speculate as to the contribution of each of the genes to this resistance phenotype. Both TEM and SHV enzymes belong to the class A family of β-lactamases and are ubiquitous among *E. coli* isolates, being frequently associated with therapeutic failure (43, 69).

The presence of $bla_{\text{CTX-M-2}}$ in a number of isolates from horses is interesting, considering the global distribution of these genes. CTX-M enzymes are currently regarded as the predominant ESβL type of animal origin, while they have also been associated with human isolates in Europe since the late 1990s (39). In Ireland, $bla_{\text{CTX-M}}$-mediated extended-spectrum β-lactam resistance is widespread, as demonstrated by a recent nationwide survey of *Enterobacteriaceae* isolates recovered from clinical cases in humans over an 11-year period (46). CTX-M group 1 followed by CTX-M group 9, represented by CTX-M-15 and CTX-M-14, respectively, are the most frequently reported examples (46). In our study, the transmissibility of $bla_{\text{CTX-M-2}}$ was demonstrated following conjugation, and this observation was consistent with our current knowledge of CTX-M enzymes, which are typically carried by multiresistance plasmids (56).

We identified the $bla_{\text{CMY-2}}$ gene in 16 isolates (2 bovine, 1 canine, and 13 equine). Consistent with the genotype, all of these isolates displayed amoxicillin-clavulanate resistance and, and in most cases, were resistant to cefpodoxime and ceftiofur. To our knowledge, neither $bla_{\text{CMY-2}}$ nor $bla_{\text{CTX-M-2}}$ has previously been reported in animals in Ireland. A growing incidence of $bla_{\text{CMY-2}}$ in recent years has been linked by some investigators to the extensive use of ceftiofur (25, 67), an expanded-spectrum veterinary cephalosporin, while others have suggested no significant impact of selection pressure on the frequency and transfer of the $bla_{\text{CMY-2}}$ genes (13). Two recent studies investigating the mechanisms of β-lactam resistance in horses identified $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{SHV-1}}$ β-lactamase-encoding genes (1, 73). However, to our knowledge, the $\text{bla}_{\text{CTX-M-2}}$ gene has never been reported in bacteria of equine origin.

Despite the fact that chloramphenicol is banned by European Union legislation from use in food-producing animals, resistance to this antimicrobial remains prevalent (61). The *cat* gene, responsible for enzymatic resistance, was found to be predominant among our isolates. Furthermore, conjugal transfer of *cat*-mediated chloramphenicol resistance associated with a high-molecular-weight plasmid was demonstrated for one equine isolate. The resulting transconjugants were also resistant to a wide array of β-lactams, aminoglycosides, sulfonamides, and tetracycline (Table 3). These data support the findings in other studies that reported chloramphenicol acetyltransferases to be frequent causes of resistance to this antimicrobial compound (47). The occurrence of *cmlA*, encoding a specific chloramphenicol transporter, was significantly lower in the collection ($n = 12$; 9%), and its transfer by conjugation was not observed. As expected, florfenicol resistance occurred concomitantly with resistance to chloramphenicol and could be linked directly to the presence of *floR* (28 of 30 Ffc-resistant strains) or *cat* (2 isolates). Florfenicol, a fluorinated derivative of chloramphenicol, is a strictly controlled veterinary drug that is licensed for use in respiratory infections in cattle and pigs (61). The *floR* gene is a common genetic determinant responsible for florfenicol resistance in Gram-negative bacteria, including animal-derived *E. coli* strains (12, 15, 30). In our study, *floR*-mediated florfenicol resistance was transferred by conjugation along with plasmids of approximately 100 to 150 kb from one equine and two canine isolates. Previous reports showed that the *floR* determinant was either chromosomally located or resided on large conjugative plasmids (6, 12, 15, 74).

Common sulfonamide resistance in the collection could be attributed to the presence of the *sul1* and *sul2* genes. Our PCR data showed that of 74 strains investigated, 56 tested positive for *sul1*, 66 tested positive for *sul2*, and only 1 carried *sul3*. Transfer of both *sul1* and *sul2* along with other resistance genes was observed. Examination of the transconjugants revealed that sulfonamide resistance was associated with large self-transmissible plasmids of approximately 100 to 150 kb and, in one case, a plasmid of 7 kb, all of which conferred a multidrug resistance phenotype (see Table 3 for details). This is in agreement with earlier studies that reported *sul1* and *sul2* to be common and occurring at similar frequencies among bacteria from the *Enterobacteriaceae* family (32, 54). The *suI1* gene is usually plasmid encoded and strongly associated with Tn*21*-like class 1 integrons, wherein it constitutes part of the 3′ conserved structure (21, 55). Thus, it is to be expected that *sul1* would be cotransferred with genetic markers such as *intI1* and *qacEΔ1*. The *sul2* marker is usually located on small plasmids of the IncQ family and also on plasmids represented by pBP1 (71, 78). Enne and coworkers, in their study, demonstrated the

transfer of a *sul2* determinant along with its association with large multiresistance plasmids in a collection of bacteria originating from the United Kingdom (17). In a more recent study, the occurrence of *sul3* was found to be limited mainly to porcine *E. coli* isolates (32). Similarly, in our study, this genetic determinant was identified in only a single porcine isolate.

Of the 6 tetracycline resistance genes analyzed by PCR, only *tet*(A) and *tet*(B) were detected in the collection, with 42% of strains carrying *tet*(A) and 50% being positive for *tet*(B). Six of these isolates possessed both determinants. Transfer of both *tet*(A) and *tet*(B) was subsequently demonstrated in mating assays with selected strains. These data supported the findings of others with respect to the prevalence of the *tet* genes. Both determinants were previously found to be particularly abundant in tetracycline-resistant *E. coli* isolates cultured from animals. However, their distribution varied in different animal species and in different geographical regions (23, 33, 35, 62).

In summary, this report showed that the occurrence of integrons among MDR *E. coli* isolates from hospitalized animals in Ireland is high (94.6%). Their contribution to resistance appears to be directed against older antimicrobial compounds, including streptomycin, trimethoprim, and sulfonamides. MDR phenotypes, including resistance to newer drugs, are transferable and in some cases directly linked to the presence of large conjugative plasmids carrying multiple resistance determinants and integrons. This observation can help to explain the coresistance to several unrelated drug classes that was detected in these isolates and, importantly, emphasizes the dangers of coselection. Colonization of hospitalized horses with multiresistant ESβL and/or AmpC-like enzymes producing *E. coli* is particularly challenging, given the importance of these drugs and the potential for their dissemination to the environment and individuals such as veterinarians and others who have direct contact with these animals. Identification of resistance genes allied to the investigation of their transfer potential among bacteria of animal origin provides valuable information regarding this important resistance gene pool and the dynamics of resistance gene exchange. Further studies are required to establish the true nature of the animal resistance reservoir.

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