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Antimicrobial Resistance Genes in Enterotoxigenic *Escherichia coli* O149:K91 Isolates Obtained over a 23-Year Period from Pigs

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A total of 112 *Escherichia coli* O149:K91 strains isolated from pigs with diarrhea in Quebec, Canada, between 1978 and 2000 were characterized for their genotypic antimicrobial resistance profiles. Tests for resistance to 10 antimicrobial agents were conducted. Resistance to tetracycline and sulfonamides was found to be the most frequent, but resistance to cefotaxime and ceftiofur was absent. An increase in the number of isolates resistant to at least three antimicrobials was observed over time. The distribution of 28 resistance genes covering six antimicrobial families (beta-lactams, aminoglycosides, phenicols, tetracycline, trimethoprim, and sulfonamides) was assessed by colony hybridization. Significant differences in the distributions of tetracycline [*tet*(A), *tet*(B), *tet*(C)], trimethoprim (*dhfrI*, *dhfrV*, *dhfrXIII*), and sulfonamide (*sulI*, *sulII*) resistance genes were observed during the study period (1978 to 2000). Sixty percent of the isolates possessed a class 1 integron, illustrating the importance of integrons in the epidemiology of antibiotic resistance in *E. coli* strains from pigs. Amplification of the integron's variable region resulted in four distinct fragments of 1, 1.3, 1.6, and 1.8 kb, with the 1.6- and 1.8-kb fragments appearing only during the last half of the study period. Examination of linkages among the different resistance genes showed a variety of positive and negative associations. Association analysis of isolates divided into two groups, those isolated between 1978 and 1989 and those isolated between 1990 and 2000, revealed the appearance of new positive resistance gene associations. Our genotypic resistance analyses of ETEC isolates from pigs indicate that many of the antibiotic resistance genes behind phenotypic resistance are not static but, rather, are in a state of flux driven by various selection forces such as the use of specific antimicrobials.

Enterotoxigenic *Escherichia coli* (ETEC) is an important pathogen of swine, causing diarrhea in newborn and postweaning pigs. ETEC serotype O149:K91 has been found more frequently in recent years and is the predominant serotype universally associated with diarrhea in pigs (3, 19, 31). ETEC strains are also associated with several other serogroups, i.e., O8, O9, O20, O101, O138, and O141 (15). Despite this limited number of serogroups, ETEC strains show diverse genetic backgrounds. With the worldwide progressive increase in antimicrobial resistance among *E. coli* isolates, treatment of diarrhea in postweaning pigs has become increasingly difficult. In Quebec, Canada, aminopenicillins, chlortetracycline, trimethoprim-sulfonamides, and, to a lesser extent, the aminoglycosides neomycin and apramycin are the usual antimicrobials used to treat diarrhea; however, with the appearance of increased antimicrobial resistance, the expanded-spectrum cephalosporin (ceftiofur) is seeing increased usage (3).

The dissemination of antibiotic resistance genes among bacterial strains is an increasing problem in infectious diseases. Many antibiotic resistance genes are located on plasmids and/or transposons, enabling their transfer among a variety of bacterial species. In recent years, another mechanism of resistance gene dissemination has been discovered. That mechanism involves a DNA element that mediates the integration of resistance genes by a site-specific recombinational mechanism. This newly recognized DNA element, called an integron, either is found as part of a transposon within the Tn*21* family or is found independently on several groups of broad-host-range plasmids. Class 1 integrons possess two conserved segments separated by a variable region (VR) which includes integrated antibiotic resistance genes or cassettes of unknown function (25). The 3′ conserved segment contains the *qacE*Δ1 and *sulI* genes and an open reading frame (ORF) called *orf5*. The *qacE*Δ1 and *sulI* genes determine resistance to ethidium bromide and quaternary ammonium compounds and resistance to sulfonamide, respectively (25).

Hence, a study to analyze the evolution of different antimicrobial resistance phenotypes in ETEC O149:K91 strains isolated from piglets with clinical cases of diarrhea and other intestinal disorders from 1978 to 2000 (F. Fontaine, N. Nadeau, S. D'Allaire, S. Péres, and J. M. Fairbrother, unpublished data) revealed an increase in the number of multiantimicrobial-resistant strains in a bacterial population with time. In the present study, these ETEC O149:K91 strains were characterized for their genotypic resistance gene profiles. Our findings show that during the 23-year study period, the antimicrobial resistance gene distribution among *E. coli* O149:K91 isolates has been dynamic and that the observed increases in

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phenotypic resistance correlate with an increase in multigene resistance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Numerous *E. coli* isolates from diseased animals are sent to The *E. coli* Laboratory at the Faculté de Médecine Vétérinaire, at Saint-Hyacinthe, Quebec, Canada, for serotyping and virulence factor determination (11). For our study, we selected all the ETEC isolates in this collection that came from pigs with diarrhea, belonged to serogroup O149:K91, contained virulence factors typically associated with the ETEC pathotype (e.g., heat-stable enterotoxins a and b, F4, and heat-labile enterotoxin), and were resistant to one of the antimicrobials for which genes for resistance were tested. In order to minimize any selection bias, only one isolate per farm was selected. The farms from which the isolates were obtained were distributed throughout the various regions of the province of Quebec. After 21 isolates which were susceptible to all antimicrobials tested were excluded, the remaining 112 ETEC isolates which were obtained over a 23-year period were divided into four groups on the basis of the year of isolation: 1978 to 1984, 1985 to 1989, 1990 to 1994, and 1995 to 2000. The reference strain, *E. coli* ATCC 25922, was used to test each lot of antimicrobial agents by the disk diffusion method. The bacterial strains, kept at −80°C in tryptic soy broth medium containing 10% glycerol, were cultured on tryptic soy agar supplemented with 5% (vol/vol) sheep blood.

The 28 strains used as positive controls and templates for DNA amplification were obtained from different laboratories (Table 1). These strains were kept at −80°C as frozen stocks 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 concentrations: 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).

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was carried out by the disk diffusion method according to the recommendations reported by the National Committee for Clinical Laboratory Standards (NCCLS) (33). As recommended by the NCCLS, Mueller-Hinton agar batches were used as the culture medium. The antimicrobial agent disks used in this study were beta-lactams (ampicillin [10 μg], ceftiofur [30 μg], cefotaxime [30 μg]); aminoglycosides (gentamicin [10 μg], kanamycin [30 μg], neomycin [30 μg]), tetracycline (30 μg), chloramphenicol (30 μg), trimethoprim (5 μg), and sulfaminoxazole (250 μg) (BBL, Bristol, Conn.).

The zone diameters around all disks except the neomycin disk were interpreted by using the recommendations of the NCCLS; the breakpoints used for neomycin were those recommended by the manufacturer. In the case of trimethoprim, the zone diameters were interpreted by the method in the NCCLS recommendations for enterobacteria from the urinary tract.

PCR primers and amplification. Resistance gene primers were designed by using the software program Prime (Genetics Computer Group, Madison, Wis.). Oligonucleotide primers were synthesized with a DNA synthesizer (BioCorp Inc., Montreal, Quebec, Canada). The PCR primers, their amplified product sizes, as well as the references for the corresponding strains used as amplification templates are listed in Table 1. The class 1 integron is characterized by the *qacE*Δ1 and *sulI* genes at its 3′ conserved segment. Primers located at the 3′ conserved segment were used as described by Sandvang and Aarestrup (27) to investigate whether the class 1 integron was present (Table 1).

Amplifications were performed with 5 μl of supernatant from bacterial preparations that had been boiled for 10 min (8). The PCR mixture (total volume, 50 μl) included 29.6 μl of H_2O, 5.0 μl of 10× PCR buffer (Amersham Pharmacia Biotech Inc., Piscataway, N.J.), 5.0 μl of 2 mM deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Amersham Pharmacia Biotech Inc.), and 25 pmol of each primer. DNA amplification was carried out in a GeneAmp PCR system 9700 (Perkin-Elmer, Foster City, Calif.) by using 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. A sample of 3 μl of the PCR product was verified for size and purity by gel electrophoresis (1.2% [wt/vol] agarose in 1× TAE [Tris-acetate-EDTA] buffer).

All isolates that contained the 3′ conserved segment of the class 1 integron were further investigated by another PCR amplification of a VR within the integron by using the following primers described by Sandvang and Aarestrup (27) (Table 1). Amplification conditions for these primers were as follows: 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 5 min.

DNA sequencing. The amplified products were purified with a QIAquick gel extraction kit (QIAgen Inc., Mississauga, Ontario, Canada). Their sequences were confirmed with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit by using a model 377 DNA sequencer (Applied Biosystems, Foster City, Calif.). Sequences were submitted to the National Center for Biotechnology Information (Bethesda, Md.) for comparison with sequences in GenBank by use of the BLAST program. Multiple DNA alignments were performed by using the CLUSTALW program (32).

Colony hybridization. The amplicons were labeled with [α-^{32}P]CTP by using a DNA labeling beads kit (Amersham Pharmacia Biotech Inc.). Colony hybridizations were performed as described previously (11).

Statistical methods. Comparisons of the associations between resistance genes in the total population were made by using Pearson's chi-square exact test (SAS, version 8.2; SAS Institute, Inc., Cary, N.C.). The statistical significance was set at a P value of <0.05. An association between two genes can be positive, indicating that the genes are found together, or negative, indicating that the genes are not found together.

RESULTS

Antimicrobial resistance phenotype characteristics. Almost all (93%) of the 112 isolates were resistant to tetracycline, and a similar number (91%) were resistant to sulfonamides. The rates of resistance to ampicillin, neomycin, kanamycin, chloramphenicol, and trimethoprim ranged from 21 to 38%, whereas only 14% of the isolates were resistant to gentamicin (Table 2). None of the isolates tested was resistant to cefotaxime or ceftiofur. Some isolates demonstrated a zone of inhibition at the limit (the zone between resistance and susceptibility) for aminoglycosides, tetracycline, and sulfonamides. To facilitate our analysis, these isolates were considered resistant in this study.

As shown in Table 2, the percentage of isolates with multiresistance to certain antimicrobials increased over time. Between 1978 and 1984, the percentage of isolates resistant to neomycin, kanamycin, tetracycline, and sulfonamides was already quite high. The rates of resistance to ampicillin, gentamicin, chloramphenicol, and trimethoprim showed the greatest increases in the last period (1995 to 2000). The proportions of strains resistant to at least three antimicrobials and isolated between 1978 and 1984, 1985 and 1989, 1990 and 1994, and 1995 and 2000 were 44, 31, 68, and 83%, respectively (data not shown).

Distributions of resistance genes among O149:K91 ETEC isolates. The choice of the resistance genes to be studied was based on their relative importance, as observed in resistant *E. coli* isolates (5, 6, 13, 20, 21, 27, 35). Therefore, 28 genes coding for resistance to antimicrobials in six families (beta-lactams, aminoglycosides, tetracycline, phenicols, trimethoprim, and sulfonamides) were chosen to determine their distributions among O149:K91 ETEC isolates from pigs with diarrhea (Table 1).

(i) Beta-lactams. The DNA hybridization probes bla_{TEM} and bla_{SHV} detect all known variants within the corresponding bla_{TEM} and bla_{SHV} gene families on colony hybridization. The $bla_{\mathrm{OXA\text{-}7}}$ probe detects bla_{OXA} variants such as $bla_{\mathrm{OXA\text{-}10}}$ to $bla_{\mathrm{OXA\text{-}14}}$, $bla_{\mathrm{OXA\text{-}16}}$ to $bla_{\mathrm{OXA\text{-}19}}$, $bla_{\mathrm{OXA\text{-}28}}$, $bla_{\mathrm{OXA\text{-}31}}$, $bla_{\mathrm{OXA\text{-}35}}$ (80 to 96% similarity), and $bla_{\mathrm{PSE\text{-}2}}$ (96.3% similarity). To further discriminate variants among the $bla_{\mathrm{OXA\text{-}7}}$ hybridization probe-positive isolates, a PCR amplification was undertaken with $bla_{\mathrm{OXA\text{-}7}}$-specific primers. All 112 isolates studied possessed at least one of the resistance genes for which tests were conducted.

Eighty-six percent of the ampicillin-resistant isolates and all bla_{SHV}-positive isolates were bla_{TEM} positive. The $bla_{\mathrm{OXA\text{-}1}}$ gene

TABLE 1. PCR primers used for antimicrobial resistance gene and class 1 integron amplifications

Antimicrobial family	Resistance gene	Forward PCR primer sequence (5′–3′)	Reverse PCR primer sequence (5′–3′)	Amplicon size (bp)	GenBank accession no.	Source of DNA
Beta-lactams	bla_{TEM}	GAGTATTCAACATTTTCGT	ACCAATGCTTAATCAGTGA	857	AF309824	R. C. Levesque
	bla_{SHV}	TCGCCTGTGTATTATCTCCC	CGCAGATAAATCACCACAATG	768	AF148850	R. C. Levesque
	bla_{OXA-1}	GCAGCGCCAGTGCATCAAC	CCGCATCAAATGCCATAAGTG	198	AJ238349	Pasteur Institute
	bla_{OXA-7}	AGTTCTCTGCCGAAGCC	TCTCAACCCAACCAACCC	591	X75562	R. C. Levesque
	bla_{PSE-4}	CTGCTCGTATAGGTGTTTCC	TCGCATCATTTCGCTCTTC	705	J05162	R. C. Levesque
	$bla_{CTX-M-3}$	AATCACTGCGTCAGTTCAC	TTTATCCCCCACAACCCAG	701	X92506	A. Huletsky
Aminoglycosides	*ant(2″)-Ia* (*aadB*)[a]	TCCAGAACCTTGACCGAAC	GCAAGACCTCAACCTTTTCC	700	X04555	R. C. Levesque
	aac(3)-IIa (*aaaC2*)	CGGAAGGCAATAACGGAG	TCGAACAGGTAGCACTGAG	740	X54723	D. Sandvang
	aac(3)-IV	GTGTGCTGCTGGTCCACAGC	AGTTGACCCAGGGCTGTCGC	627	X01385	J. Harel
	apk(3′)-Ia (*aphA1*)	ATGGGCTCGCGATAATGTC	CTCACCGAGGCAGTTCCAT	600	M18329	J. Harel
	aph(3′)-IIa (*aphA2*)	GAACAAGATGGATTGCACGC	GCTCTTCAGCAATATCACGG	680	V00618	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
	tet(D)	TGGGCAGATGGTCAGATAAG	CAGCACACCCTGTAGTTTTC	827	X65876	S. B. Levy
	tet(E)	TTAATGGCAACAGCCAGC	TCCATACCCATCCATTCCAC	853	L06940	M. C. Roberts
	tet(Y)	ACCGCACTCATTGTTGTC	TTCCAAGCAGCAACACAC	823	AF070999	M. C. Roberts
Phenicols	*catI*	AGTTGCTCAATGTACCTATAACC	TTGTAATTCATTAAGCATTCTGCC	547	M62822	J. Harel
	catII	ACACTTTGCCCTTTATCGTC	TGAAAGCCATCACATACTGC	543	X53796	Pasteur Institute
	catIII	TTCGCCGTGAGCATTTTG	TCGGATGAGTATGGGCAAC	286	X07848	I. A. Murray
	floR	CGCCGTCATTCCTCACCTTC	GATCACGGGCCACGCTGTGTC	215	AF252855	D. G. White
Trimethoprim	*dhfrI*	AAGAATGGAGTTATCGGGAATG	GGGTAAAAACTGGCCTAAAATTG	391	X00926	J. Harel
	dhfrV	CTGCAAAAGCGAAAAACGG	AGCAATAGTTAATGTTTGAGCTAAAG	432	X12868	O. Sköld
	dhfrVII	GGTAATGGCCCTGATATCCC	TGTAGATTTGACCGCCACC	265	X58425	O. Sköld
	dhfrIX	TCTAAACATGATTGTCGCTGTC	TTGTTTTCAGTAATGGTCGGG	462	X57730	C. Wallen
	dhfrXIII	CAGGTGAGCAGAAGATTTTT	CCTCAAAGGTTTGATGTACC	294	Z50802	P. V. Adrian
Sulfonamides	*sulI*	TTCGGCATTCTGAATCTCAC	ATGATCTAACCCTCGGTCTC	822	X12869	R. C. Levesque
	sulII	CGGCATCGTCAACATAACC	GTGTGCGGATGAAGTCAG	722	M36657	J. Harel
Class 1 integron	*qacEΔI-sulI*	ATCGCAATAGTTGGCGAAGT	GCAAGGCGGAAACCCGCGCC	797	X12870	
	VR	GGCATCCAAGCAGCAAGC	AAGCAGACTTGACCTGAT	Variable	X12870	

[a] Alternative nomenclature.

TABLE 2. Trends in resistance of ETEC O149:K91 isolates with time, as determined by the disk diffusion method

ATM[b]	No. (%) of resistant ETEC O149:K91 isolates[a]				
	1978–1984 (n = 25)	1985–1989 (n = 24)	1990–1994 (n = 22)	1995–2000 (n = 41)	Total (n = 112)
AMP	8 (32)	2 (8)	8 (36)	25 (61)	43 (38)
CTX	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
XLN	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
GEN	1 (4)	2 (8)	1 (4)	12 (29)	16 (14)
NEO	10 (40)	5 (21)	4 (18)	20 (49)	39 (35)
KAN	10 (40)	6 (25)	8 (36)	19 (46)	43 (38)
TET	22 (88)	22 (92)	21 (95)	39 (95)	104 (93)
CHL	4 (16)	1 (4)	2 (9)	17 (41)	24 (21)
TMP	1 (4)	2 (8)	2 (9)	25 (61)	30 (27)
SUL	22 (88)	24 (100)	22 (100)	34 (83)	102 (91)

[a] Phenotypic resistance was overestimated because only isolates showing initial resistance to one antimicrobial were further analyzed.
[b] ATM, antimicrobials; AMP, ampicillin; CTX, cefotaxime; XLN, ceftiofur; GEN, gentamicin; NEO, neomycin; KAN, kanamycin; TET, tetracycline; CHL, chloramphenicol; TMP, trimethoprim; SUL, sulfonamides.

was found in only 25% of the ampicillin-resistant strains isolated during the third period (1990 to 1994). None of the isolates tested were positive for hybridization with the $bla_{\text{CTX-M-3}}$, $bla_{\text{PSE-4}}$, or $bla_{\text{OXA-7}}$ probe. One of the 69 isolates susceptible to beta-lactams hybridized with bla_{TEM}, and four isolates resistant to ampicillin did not possess any of the beta-lactam resistance genes for which tests were conducted.

(ii) Aminoglycosides. Of the five aminoglycoside resistance genes for which tests were conducted, only *aph*(3′)-*Ia*, *aph*(3′)-*IIa*, and *aac*(3)-*IV* were found among the resistant isolates (Table 3). The *aph*(3′)-*Ia* and *aph*(3′)-*IIa* genes, encode a kanamycin and a neomycin resistance phenotype, and these genes were found, respectively, in 87 and 15% of the neomycin-resistant isolates, and in 79 and 19% of the kanamycin-resistant isolates. The relative importance of *aph*(3′)-*Ia* and *aph*(3′)-*IIa* varied throughout the studied periods (1978 to 2000). Two isolates harboring the *aph*(3′)-*Ia* gene were susceptible to kanamycin and neomycin. Only one gentamicin resistance gene, *aac*(3)-*IV*, was found among the isolates. Seventy-five percent of the gentamicin-resistant isolates and two gentamicin-susceptible isolates had this gene.

(iii) Tetracycline. Of the six tetracycline resistance genes targeted, only the *tet*(A), *tet*(B), and *tet*(C) genes were detected. The *tet*(B) gene was found in 80% of the tetracycline-resistant isolates and was by far the gene found the most frequently during the first three periods. Only 25% tetracycline-resistant isolates were positive for hybridization with the *tet*(A) and *tet*(C) probes over the whole study period. These two genes, which were less prevalent in the first periods (<15%), appeared even slightly more frequently (54%) than *tet*(B) (51%) between 1995 and 2000 (Table 3). An association between the *tet*(A) and the *tet*(C) genes was observed in all *tet*(A)-positive isolates, and only 5% of the *tet*-positive isolates possessed all three tetracycline resistance genes. No link be-

TABLE 3. Distribution of antimicrobial resistance genes detected in ETEC O149:K91 isolates by isolation period

Antimicrobial to which resistance was detected	Antimicrobial resistance probe	No. (%)[a] of positive isolates by isolation period				
		1978–1984	1985–1989	1990–1994	1995–2000	Total
Ampicillin	bla_{TEM}	8 (100)	1 (50)	3 (38)	25 (100)	37 (86)
	bla_{SHV}	3 (38)	0 (0)	1 (12)	5 (20)	9 (21)
	$bla_{\text{OXA-1}}$	0 (0)	0 (0)	2 (25)	0 (0)	2 (5)
	$bla_{\text{OXA-7}}$, bla_{PSE}, $bla_{\text{CTX-M-3}}$	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamicin	*aac*(3)-*IV*	0 (0)	0 (0)	1 (100)	11 (92)	12 (75)
	ant(2″)-*Ia*, *aac*(3)-*IIa*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Neomycin	*aph*(3′)-*Ia*	9 (90)	4 (80)	4 (100)	17 (85)	34 (87)
	aph(3′)-*IIa*	1 (10)	1 (20)	2 (50)	2 (10)	6 (15)
	ant(2″)-*Ia*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Kanamycin	*aph*(3′)-*Ia*	9 (90)	4 (67)	4 (50)	17 (89)	34 (79)
	aph(3′)-*IIa*	1 (10)	2 (33)	3 (38)	2 (10)	8 (19)
	ant(2″)-*Ia*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Tetracycline	*tet*(A)	1 (4)	1 (4)	3 (14)	21 (54)	26 (25)
	tet(B)	21 (95)	23 (100)	19 (90)	20 (51)	83 (80)
	tet(C)	1 (4)	1 (4)	3 (14)	21 (54)	26 (25)
	tet(D), *tet*(E), *tet*(Y)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Chloramphenicol	*catI*	4 (100)	1 (100)	2 (100)	12 (70)	19 (79)
	catII, *catIII*, *floR*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trimethoprim	*dhfrI*	0 (0)	2 (100)	0 (0)	4 (16)	6 (20)
	dhfrV	0 (0)	0 (0)	0 (0)	14 (56)	14 (47)
	dhfrXIII	0 (0)	0 (0)	2 (100)	7 (28)	9 (30)
	dhfrVII, *dhfrIX*	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Sulfonamides	*sulI*	18 (82)	20 (83)	21 (95)	22 (65)	81 (79)
	sulII	8 (36)	7 (29)	3 (14)	19 (56)	37 (36)

[a] Percentage of probe-positive isolates among resistant ETEC isolates by period.

TABLE 4. Distribution of class 1 integron among the ETEC O149:K91 isolates

Isolation period	Total no. of isolates	No. of integron class 1-positive isolates	% of integron class 1-positive isolates by VR length			
			1.0 kb	1.3 kb	1.6 kb	1.8 kb
1978–1984	25	14	21	79	0	0
1985–1989	24	14	29	72	0	0
1990–1994	22	19	26	68	0	6
1995–2000	41	20	35	15	35	15
Total	112	67	29	55	10	6

tween these genes and phenotype was observed in four isolates: 2 of the 8 isolates susceptible to tetracycline were *tet*(B) positive, and 2 of the 104 tetracycline-resistant isolates were negative for the six *tet* genes for which tests were conducted.

(iv) Phenicols. Only the chloramphenicol resistance genes *catI* and *floR* were detected among the isolates tested. The *catI* gene was found in 79% of the chloramphenicol-resistant isolates. The *floR* gene was not detected in chloramphenicol-resistant isolates. Three chloramphenicol-susceptible isolates were positive for hybridization with the *catI* probe, and one strain isolated in 1989 was *floR* positive but chloramphenicol susceptible. Four of the 24 chloramphenicol-resistant isolates did not hybridize with the *cat* genes for which tests were conducted.

(v) Trimethoprim. In our study, the trimethoprim resistance phenotype was found to be associated with the presence of three genes, *dhfrI*, *dhfrV*, and *dhfrXIII*. These genes were not detected in strains isolated from 1978 to 1984. The *dhfrI* gene was detected in strains isolated in the second period (1985 to 1989), whereas the *dhfrXIII* gene appeared in the third period (1990 to 1994). All three trimethoprim resistance genes were found among the strains isolated in the last period, with a predominance of *dhfrV*. Among the trimethoprim-resistant isolates, three were negative for the genes for which tests were conducted, whereas one susceptible isolate was found to be *dhfrXIII* positive.

(vi) Sulfonamides. Seventy-nine percent of the sulfonamide-resistant isolates possessed the *sulI* gene, whereas 36% possessed *sulII* (Table 3). Seventeen percent of these resistant isolates possessed both *sulI* and *sulII*. The percentage of the isolates positive for *sulI* did not vary significantly during any of the periods. However, the percentage of *sulII*-positive isolates increased significantly to 56% in the last period. No correlation between the presence of sulfonamide resistance genes and phenotype was observed for six isolates. Four of the 10 sulfonamide-susceptible isolates were *sulI* positive, whereas 2 of the 102 sulfonamide-resistant isolates were found to be *sulI* and *sulII* negative.

Identification of integrons. Of the 112 isolates, a fragment from the class 1 integron 3′ conserved region was amplified from 67 (60%) isolates by PCR (Table 4). Among these positive isolates, 84% were also positive for hybridization with a *sulI* probe by colony hybridization (data not shown). Among the 67 class 1 integron-positive isolates, four distinct amplicons of 1, 1.3, 1.6, and 1.8 kb were obtained by amplification of the VR by using the primers Fint1 and Rint1. More specifically, a 1.3-kb VR fragment was amplified from more than half of the isolates (55%), followed by amplification of a 1-kb fragment from 29% of the isolates. The remaining 16% of the isolates produced fragments larger than 1.3 kb. The percentage of isolates from which VR fragments larger than 1.6 and 1.8 kb were amplified increased over time. Before 1990, none of the class 1 integron-positive isolates had a VR fragment larger than 1.3 kb. However, a larger VR fragment appeared in one (6%) such isolate in the period from 1990 to 1994, and the frequency of isolates with a larger VR fragment increased to 50% in the latest period (1995 to 2000).

Two representative VR fragments of each length were sequenced. Both VR fragments of the same length contained the same gene cassettes with more than 95% similarity. The 1-kb VR fragment contained the *ant*(*3″*)-*Ia* (*aadA1*) gene cassette (GenBank accession no. X12870) encoding streptomycin and spectinomycin resistance. The most frequently observed VR amplified fragments (i.e., fragments of 1.3 kb) contained, in addition to the *ant*(*3″*)-*Ia* gene cassette found in the 1-kb fragment, an ORF named *orfD* (GenBank accession nos. M86913 and AF140629, respectively). The 1.6-kb VR fragments contained two gene cassettes, *dhfrIb*, encoding resistance to trimethoprim, and *ant*(*3″*)-*If* (*aadA6*), encoding resistance to streptomycin and spectinomycin (GenBank accession nos. AF393510 and AF140629, respectively). Finally, the 1.8-kb VR fragments possessed two gene cassettes, *dhfrXII* and *ant*(*3″*)-*If*, which confer resistance to trimethoprim and resistance to streptomycin and spectinomycin, respectively, and one ORF named *orfF* (GenBank accession nos. Z21672 and AF284063, respectively).

Association between resistance genes. In order to determine whether possible associations exist between the resistance genes found among our isolates and whether the coappearance of some resistance genes could be confirmed statistically, an analysis of association was done by using Pearson's chi-square exact test. Significant associations ($P < 0.05$) with respect to the occurrence of individual resistance genes among the whole collection of ETEC isolates were detected (Table 5). Some positive associations were obvious, for example, the association of the bla_{TEM} and bla_{SHV} genes as well the association of the *tet*(A) and *tet*(C) genes. The bla_{TEM} gene was associated with the *aph*(*3′*)-*Ia*, *aac*(*3*)-*IV*, *dhfrV*, and *catI* genes and also, although less strongly, with the *sulII* gene (Table 5). The *aac*(*3*)-*IV* gene showed a positive association with the bla_{TEM} and bla_{SHV} genes and also with the *aph*(*3′*)-*Ia*, *dhfrV*, and *dhfrXII* genes. The *aph*(*3′*)-*IIa* gene was positively associated with the *tet*(A) and *tet*(C) genes but was negatively associated with the *tet*(B) gene. In contrast, the *sulI* gene was positively associated with the *tet*(B) gene but was negatively associated with the *tet*(A) and *tet*(C) genes. Although the class 1 integron was found together with the *sulI* gene in 68% of the *sulI*-positive isolates, no statistically significant association was observed between the presence of the class 1 integron and *sulI*. This absence of a correlation is probably due to the presence of *sulI* in integron-negative isolates.

The association analysis was subsequently performed with two groups of the collection of isolates, with group 1 representing those strains isolated between 1978 and 1989 and group 2 representing those strains isolated between 1990 and 2000 (data not shown). New positive associations were observed for strains isolated from 1990 to 2000, such as bla_{TEM}

TABLE 5. Association between various antimicrobial resistance genes and class 1 integron among ETEC O149:K91 isolates

Resistance gene	Significance of association of antimicrobial resistance gene and class 1 integron[a]													
	bla$_{TEM}$	*bla*$_{SHV}$	*aph(3′)-Ia*	*aph(3′)-IIa*	*aac(3)-IV*	*dhrfI*	*dhrfV*	*dhfrXIII*	*sulI*	*sulII*	*tet*(A)	*tet*(B)	*tet*(C)	*catI*
bla$_{SHV}$	+++													
aph(3′)-Ia	+++	++												
aph(3′)-IIa	−	−	−											
aac(3′)-IV	+++	+	++	−										
dhfrI	−	−	−	−	−									
dhfrV	++	++	−	−	+++	−								
dhfrXIII	−	−	−	−	+	−	−							
sulI	−	−	−	−	−	−	−	−						
sulII	+	−	−	−	−	−	−	++	−					
tet(A)	−	−	−	+	−	−	+	−	(++)	−				
tet(B)	−	−	−	(+)	−	−	−	−	+++	−	(+++)			
tet(C)	−	−	−	+	−	−	+	−	(++)	−	+++	(+++)		
catI	+++	−	−	−	+	−	++	−	−	−	−	−	−	
Class 1 integron	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a] Only the results for antimicrobial resistance genes that exhibited an association with another gene at a P level of <0.05 are shown. Significance level of association (as assessed by the chi-square exact test): −, $P > 0.05$; +, $0.05 \geq P \geq 0.01$; ++, $0.01 \geq P \geq 0.001$; +++, $P \leq 0.001$. Parentheses indicate negative associations.

with *bla*$_{SHV}$; *sulI* with *tet*(A), *tet*(B), *tet*(C), and the class 1 integron; and finally, *catI* with the class 1 integron. For strains isolated from 1990 to 2000, some positive associations appeared to be more closely linked than the associations observed for the whole collection, for example, *bla*$_{TEM}$ with *aph(3′)-Ia* and *aac(3)-IV* and *dhfrXIII* with *sulII*.

DISCUSSION

ETEC is the most common bacterial etiologic agent of diarrhea in neonatal and postweaning pigs. Although treatment of enteric *E. coli* infection in swine commonly includes the use of antimicrobials (2, 10), relatively few studies have been directed toward the characterization of the genotypic resistance profiles of *E. coli* strains isolated from animals. One study characterized the tetracycline and sulfonamide resistance gene profiles of *E. coli* strains isolated from animals and humans (17), while a second one (27) characterized the aminoglycoside resistance gene profiles of porcine and bovine *E. coli* isolates. In the present study, we characterized the gene profiles of ETEC O149:K91 strains isolated from pigs between 1978 and 2000 for detection of acquired resistance to 10 different antimicrobial agents.

In this study, phenotypic resistance was overestimated because only isolates showing initial resistance to one antimicrobial were further analyzed. Nevertheless, it remains that the phenotypic resistance observations reported here reflect the general trend observed with *E. coli* strains isolated from pigs (3, 19, 31). Although we did not test for resistance to all the antimicrobials which could inhibit the growth of *E. coli* (other aminoglycosides, quinolones or fluoroquinolones, colistin), most of the resistant isolates were resistant to more than one antimicrobial, especially during the last period (1995 to 2000). No resistance to cephalosporins (ceftiofur and cefotaxime) was observed.

Other groups (17, 31) have also noted in pig isolates the resistance to tetracycline and sulfonamide antimicrobials observed in most of our ETEC isolates. It is not surprising that the level of resistance of the isolates to these antimicrobials was so high, as these antimicrobials have been and are still being used as growth promoters in Canada and the United States, for disease prevention, and as therapy in swine production (12). Among the tetracycline-resistant isolates, the *tet*(B) gene was largely predominant until 1994, when two other closely associated tetracycline resistance genes, *tet*(A) and *tet*(C), became dominant during the last study period (1995 to 2000). Another study with pigs from three herds with different histories of antimicrobial exposure produced similar results, in that *tet*(B) was predominant in two of the herds exposed to antimicrobials, and when they were present, *tet*(A) and *tet*(C) were also found together (18). In contrast, a recent study by Lanz et al. (17) showed that the *tet*(A) gene alone is the most prevalent *tet* gene among *E. coli* isolates from pigs with diarrhea or enterotoxemia. The modes of action as well as the specificities of certain antimicrobial enzymes could exert positive selection pressure and contribute to the emergence of new genes over time. For example, class A, B, and C tetracycline resistance determinants are efflux pumps with different specificities. Most of the efflux proteins confer resistance to tetracycline but not to the minocyline or glycylcycline antimicrobial group. In contrast, the *tet*(B) gene encodes an efflux protein which confers resistance to tetracycline, doxycycline, and minocycline but not glycylcycline (24). These specificities correlate with the emergence of the diverse distribution of different tetracycline resistance genes over time. It is not known if such a selective effect exists between the commonly used tetracyclines in swine production, i.e., oxytetracycline and chlortetracycline. Similarly, the *sulI* gene was more predominant than *sulII* in strains isolated during the three first periods, whereas the *sulII* gene appeared as frequently as *sulI* in the last period (1995 to 2000). Lanz et al. (17) also observed the predominance of *sulI* among pig isolates. The *sulI* and *sulII* genes encode dihydropteroate synthase enzymes with different sensitivities (K_i values), even if the two enzymes show the same low K_m values (0.6 μM) for *p*-aminobenzoic acid, which is implicated in bacterial folic acid biosynthesis. The enzyme encoded by *sulII* discerns the normal *p*-aminobenzoic acid substrate from the inhibitor, the sulfonamides (29).

Despite a ban on the use of chloramphenicol in food animals in Canada since 1980 (9), an increase in the rate of chloram-

phenicol resistance among ETEC isolates was observed. Other investigators have also observed the persistence or an increase in the rate of chloramphenicol resistance among *E. coli* isolates from swine (3, 17, 31) and other animal species (34). Resistance to chloramphenicol was closely associated with the presence of the *catI* gene. In a study done by Bischoff et al. (3), only 4 of 48 chloramphenicol-resistant isolates harbored the *catII* gene; a relatively unknown gene, *cmlA*, was responsible for the resistance of the other isolates. Finally, we detected the *floR* gene in only one isolate. Other studies have reported the presence of *floR* in a large number of *E. coli* strains from chickens and cattle (7, 16, 35).

Interestingly, 35% of the isolates were resistant to neomycin and 38% were resistant to kanamycin, even though kanamycin is not used in the Canadian swine industry. It is likely the result of the cross-resistance caused by most of the aminoglycoside resistance genes (34). Our study showed that certain antimicrobial resistance genes were more prevalent than others and that this incidence among the ETEC isolates changed over time. The bla_{TEM} genes were widely distributed, whereas the bla_{OXA} genes appeared infrequently. Among the genes in our ETEC isolates for which tests were conducted, only the aminoglycoside resistance genes revealed limited diversity, with the most prevalent genes being *aph*(*3′*)-*Ia* and *aph*(*3′*)-*IIa*. These genes, which are also responsible for cross-resistance to different aminoglycosides (neomycin and kanamycin), did not vary in frequency over the duration of our study. In contrast, a Danish publication detected the aminoglycoside resistance genes at various frequencies, with *ant*(*2″*)-*Ia* and *aac*(*3*)-*IIa* being the most prevalent among pig isolates (27).

One clear observation arising from our study is that the number and diversity of genes driving the phenotypic resistance are dynamic. During the 23-year period, some genes or associations of genes appeared, whereas other genes became more rare (Table 5). The association of the bla_{SHV} gene with bla_{TEM} could be explained by the fact that the bla_{SHV} gene might have been acquired in isolates harboring bla_{TEM}, resulting in an increase in the rate of resistance to beta-lactams (4). We observed that the *tet*(A) and *tet*(C) genes were always found together. During the three first periods, *tet*(B) was the most prevalent tetracycline resistance gene. An incompatibility of the plasmids carrying tetracycline resistance determinants could explain the existence of the negative associations between *tet*(A)-*tet*(C) and *tet*(B) (15).

Resistance genes are associated with mobile DNA such as plasmids, transposons, and integrons, which facilitate resistance gene distribution (14, 30). Most of the isolates (62%) possessed a class 1 integron. Because integrons are characterized by their integration of many different gene cassettes between insertion sites in the VR, site-specific insertion represents another mechanism driving the evolution of the plasmids and transposons of gram-negative bacteria. Most class 1 integrons in the *E. coli* isolates in our collection contained VRs of 1.3 and 1 kb. The VRs of integrons found in Shiga toxin-producing *E. coli* isolates were of similar sizes (36). Interestingly, an increase in the VR length was observed in *E. coli* strains isolated during the last period, a phenomenon also observed by Schmitz et al. (28). They showed that the VRs of the class 1 integrons in human *E. coli* strains isolated in 1993 ranged from 0.65 to 1.8 kb and that those in human *E. coli* strains isolated in 1999 ranged from 0.75 to 3 kb, suggesting an accumulation of gene cassettes inserted in the class 1 integron among the ETEC isolates due to selection by antimicrobials. In this study, most of the isolates which possessed VRs of the same length appeared to have acquired the same gene cassettes in their integrons. However, the genes responsible for resistance to beta-lactams, tetracycline, and chloramphenicol were not associated with class 1 integrons.

Multiple cassette insertions and more than 40 distinct cassettes have been identified among integrons (25). VRs of four different sizes were detected among the class 1 integrons of the isolates in the present study (Table 4). Representatives of these VRs were sequenced. As in other studies, our study shows that the different gene cassettes of the integron VRs included genes encoding aminoglycoside and/or trimethoprim resistance, which are the most frequently described antimicrobial resistance gene cassettes (1, 23, 26).

In the last period (1995 to 2000), we observed an increase in the number of isolates with multiple resistance genes as well as the appearance of resistance genes such as *aac*(*3*)-*IV*, *dhfrV*, and *sulII*. These observed increases in the numbers of genes for resistance to the different antimicrobials are presumably due to the increased selection pressure resulting from changed management styles, for example, the early medicated weaning of piglets introduced in the early 1990s. The direct use of antimicrobials can drive the coselection of resistance genes. For example, 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 sulfisoxazole (22). In our study, the association of *aac*(*3*)-*IV*, *catI*, and *dhfrV*, which encode resistance to gentamicin, chloramphenicol, and trimethoprim, respectively, was observed; and the incidence of these genes increased with similar distributions over time. This suggests that the increased use of gentamicin or sulfamethoxazole-trimethoprim in pig production could have coselected for resistance to chloramphenicol, thus explaining the increase in chloramphenicol-resistant isolates, even though this antimicrobial agent has not been used in swine production since 1980.

In conclusion, our genotypic resistance analysis of ETEC isolates shows that the genes behind phenotypic resistance are not static but, rather, are in a state of flux driven by various selection forces such as the use of specific antimicrobials. Often, more than one gene was associated with a given phenotypic resistance. A different distribution of resistance genes was observed over time, with an increase in multigene resistance correlating with the observed phenotypic multiresistance among ETEC O149:K91 strains. The difference in the results of this study from those of studies in other countries suggests that relative resistance gene frequencies not only vary within a population over time but also vary between populations of different geographical origins. This study reinforces the necessity of using genotypic resistance analyses in future epidemiological studies.

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