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Research Note

Detection of Multiple-Antimicrobial Resistance and Characterization of the Implicated Genes in *Escherichia coli* Isolates from Foods of Animal Origin in Tunis

AHLEM JOUINI,[1,2] KARIM BEN SLAMA,[1] YOLANDA SÁENZ,[2] NAOUEL KLIBI,[1] DANIELA COSTA,[2] LAURA VINUÉ,[2] MYRIAM ZARAZAGA,[2] ABDELLATIF BOUDABOUS,[1] AND CARMEN TORRES[2]*

[1]*Laboratoire MBA, Département de Biologie, Faculté de Sciences de Tunis, Campus Universitaire, 2092 Tunis, Tunisia; and*
[2]*Área de Bioquímica y Biología Molecular, Universidad de La Rioja, 26006 Logroño, Spain*

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ABSTRACT

Phenotypic and genotypic characterization of antimicrobial resistance was conducted for 98 *Escherichia coli* isolates recovered from 40 food samples of animal origin (poultry, sheep, beef, fish, and others) obtained in supermarkets and local butcheries in Tunis during 2004 and 2005. Susceptibility to 15 antimicrobial agents was tested by disk diffusion and agar dilution methods, the mechanisms of resistance were evaluated using PCR and sequencing methods, and the clonal relationship among isolates was evaluated using pulsed-field gel electrophoresis. High resistance was detected to tetracycline, sulphonamides, nalidixic acid, ampicillin, streptomycin, and trimethoprim-sulfamethoxazole (29 to 43% of isolates), but all isolates were susceptible to cefotaxime, ceftazidime, cefoxitin, azthreonam, and amikacin. One-third of the isolates had multiresistant phenotypes (resistance to at least five different families of antimicrobial agents). Different variants of bla_{TEM}, *tet, sul, dfrA, aadA,* and *aac(3)* genes were detected in most of the strains resistant to ampicillin, tetracycline, sulphonamide, trimethoprim, streptomycin, and gentamicin, respectively. The presence of class 1 and class 2 integrons was studied in 15 sulphonamide-resistant unrelated *E. coli* strains, and 14 of these strains harbored class 1 integrons with five different arrangements of gene cassettes, and a class 2 integron with the *dfrA1 + sat + aadA1* arrangement was found in one strain. This study revealed the high diversity of antimicrobial resistance genes, some of them included in integrons, in *E. coli* isolates of food origin.

Escherichia coli, a common inhabitant of the intestinal tract of humans and animals *(29, 32),* can be easily disseminated in different ecosystems through water supplies and the food chain *(21).* In addition, this microorganism can be implicated in human infections, mainly in immunocompromised patients *(12).*

In recent years, antimicrobial resistance in human and veterinary medicine has become an area of concern because of the association between resistance and the failure of treatments used for infectious diseases. Antimicrobial agents exert selective pressure on both pathogenic bacteria and commensal microorganisms found in the intestinal tract of humans and animals, and resistant commensal bacteria could become reservoirs of antimicrobial resistance genes that could be passed to pathogenic bacteria *(8, 20, 37, 39).*

Raw meat can be contaminated with animal intestinal bacteria such as *E. coli* during slaughtering and processing, and if these microorganisms contain antimicrobial resistance genes, resistant bacteria can be transmitted through the meat to humans *(33, 34, 39).* Elaborated food products are subjected to different processes to reduce the level of bacterial contamination *(26).*

The transference of *E. coli,* including antimicrobial-resistant isolates, between animals and humans can occur via the food chain *(13, 36, 39).* The coexistence of antimicrobial resistance genes on mobile elements such as plasmids, transposons, and integrons facilitates the rapid dissemination of these genes among bacteria *(18, 30). E. coli* strains are highly capable of acquiring and transferring antimicrobial resistance genes *(40).* For this reason, it is important to know the phenotype and the genes implicated in antimicrobial resistance for bacteria (especially *E. coli*) that colonize food products and can act as reservoir of antimicrobial resistance genes. *E. coli* is considered an indicator bacterium that can be used to track the evolution of antimicrobial resistance in different ecosystems.

Studies carried out in some countries in recent years have indicated a high prevalence of antimicrobial resistance in *E. coli* strains of food origin *(4, 5, 9, 13, 24, 27).* Nevertheless, with the exception of one previous report in which extended-spectrum β-lactamases were characterized in *E. coli* isolates of food origin *(14),* these studies have not been performed in Tunisia. Our objectives in the present

* Author for correspondence. Tel: 34-941299750; Fax: 34-941299721; E-mail: carmen.torres@unirioja.es.

TABLE 1. *Type and origin of food samples analyzed and* E. coli *isolates recovered*

Type and origin of sample	No. of samples analyzed		No. of samples containing *E. coli*		Total no. of *E. coli* isolates recovered
	Supermarket	Local butchery	Supermarket	Local butchery	
Fresh food products	18	8	15	7	80
Poultry (chicken or turkey)	5	2	3	2	19
Sheep	4	4	4	4	29
Beef	2	2	1	1	8
Pork	1		1		5
Fish	3		3		11
Other (ostrich, camel, and horse)	3		3		8
Elaborated food products	11	3	2	3	18
Hamburger (chicken or turkey)	2		2		9
Salami (poultry)	4	1		1	4
Salami (beef, horse, and pork)	5	2		2	5

work were to analyze the phenotypes of antimicrobial-resistant *E. coli* isolates recovered from raw and elaborated food products in Tunis, to characterize the implicated resistance genes, and to study the possible inclusion of these genes into the more complex structures of integrons. Another objective was to determine whether antimicrobial resistance genes and their genetic supports found in food isolates were similar to those previously described in human isolates.

MATERIALS AND METHODS

Samples and *E. coli* isolation. Forty samples of food of animal origin (26 samples of raw food products and 14 samples of elaborated food products) were collected between November 2004 and April 2005 from 11 supermarkets (corresponding to the four main suppliers that distribute food in Tunis) and eight local butcheries (Table 1). Refrigerated samples were transported to the laboratory, and all samples were tested within 24 h of collection. A 30-g portion of each sample was vigorously homogenized with 270 ml of buffered peptone water and incubated for 6 h at 42°C, and 100 μl of the suspension was streaked onto MacConkey agar plates (Oxoid, Ltd., Basingstoke, UK) and incubated for 24 h at 42°C. A maximum of five suspected *E. coli* colonies from each sample (based on colony size and morphology) were selected for identification by biochemical tests and specific PCR amplification of the *uidA* gene *(11).* Isolates identified as *E. coli* were included in this study and further characterized.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was conducted on Mueller-Hinton agar plates (Difco, Becton Dickinson, Sparks, MD) by the agar disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) criteria *(6).* The antimicrobial agents tested were ampicillin (10 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), aztreonam (30 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), streptomycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), sulphonamides (200 μg), trimethoprim-sulfamethoxazole (1.25 and 23.75 μg), tetracycline (30 μg), and chloramphenicol (30 μg). The MICs of nalidixic acid and ciprofloxacin also were determined by the agar dilution method in all quinolone-resistant *E. coli* isolates *(6). E. coli* ATCC 25922 was used as a control strain.

Clonal relationship of the isolates. The clonal relationship among *E. coli* isolates was determined by pulsed-field gel electrophoresis (PFGE) using the *Xba*I enzyme as previously described *(1).* A lambda DNA ladder (New England BioLabs, Beverly, MA) with a size range of 48.5 kb to 1 Mb was used as a molecular size standard. PFGE patterns were checked both visually and by numerical analysis after conversion and normalization. Band pattern similarity analysis was performed using GEL-Pro 3.1 software. Bands whose molecular weights differed by less than 2% were considered identical. Similarities between profiles were calculated using the Dice coefficient. PFGE patterns were clustered by the unweighted pair group method using arithmetic averages as determined with the MVSP 3.13I software program. A cutoff value of 75% identity was defined.

Detection of antimicrobial resistance genes. The presence of genes associated with ampicillin (bla_{TEM}, bla_{SHV}, and bla_{OXA}), tetracycline (*tet*(A) and *tet*(B)), streptomycin (*aadA1* and *aadA2*), sulphonamides (*sul1, sul2,* and *sul3*), and gentamicin resistance [*aac(3)*-I, *aac(3)*-II, and *aac(3)*-IV) was determined using a PCR method *(23),* which in all cases included positive and negative controls from the collection of the University of La Rioja. The detection of *dfr* genes implicated in trimethoprim resistance was performed by PCR–restriction fragment length polymorphism analysis *(23).* The bla_{TEM} amplicons were sequenced to determine the type of β-lactamase gene. Both the *gyrA* and *parC* genes were amplified by PCR and sequenced in all quinolone-resistant *E. coli* isolates *(25),* and the obtained sequences were compared with those previously reported for *gyrA* (GenBank accession no. X06373) and *parC* (GenBank accession no. M58408 with the modification included in L22025).

Detection and characterization of integrons. The presence of *intI1* and *intI2* genes (encoding class 1 and class 2 integrases, respectively) was examined by PCR in all sulphonamide-resistant isolates that had unrelated PFGE patterns and also in isolates with indistinguishable PFGE patterns but different antimicrobial resistance profiles. The variable regions of class 1 and class 2 integrons were amplified by PCR and sequenced in all *intI1*- or *intI2*-positive isolates, respectively, and sequences were compared with those in GenBank to identify gene cassettes. The presence of *qacEΔ1-sul1* genes in the 3′ conserved region of class 1 integrons also was investigated in all *intI1*-positive isolates *(23).*

RESULTS AND DISCUSSION

***E. coli* recovery from food samples.** *E. coli* isolates were recovered from 27 of the 40 food samples analyzed

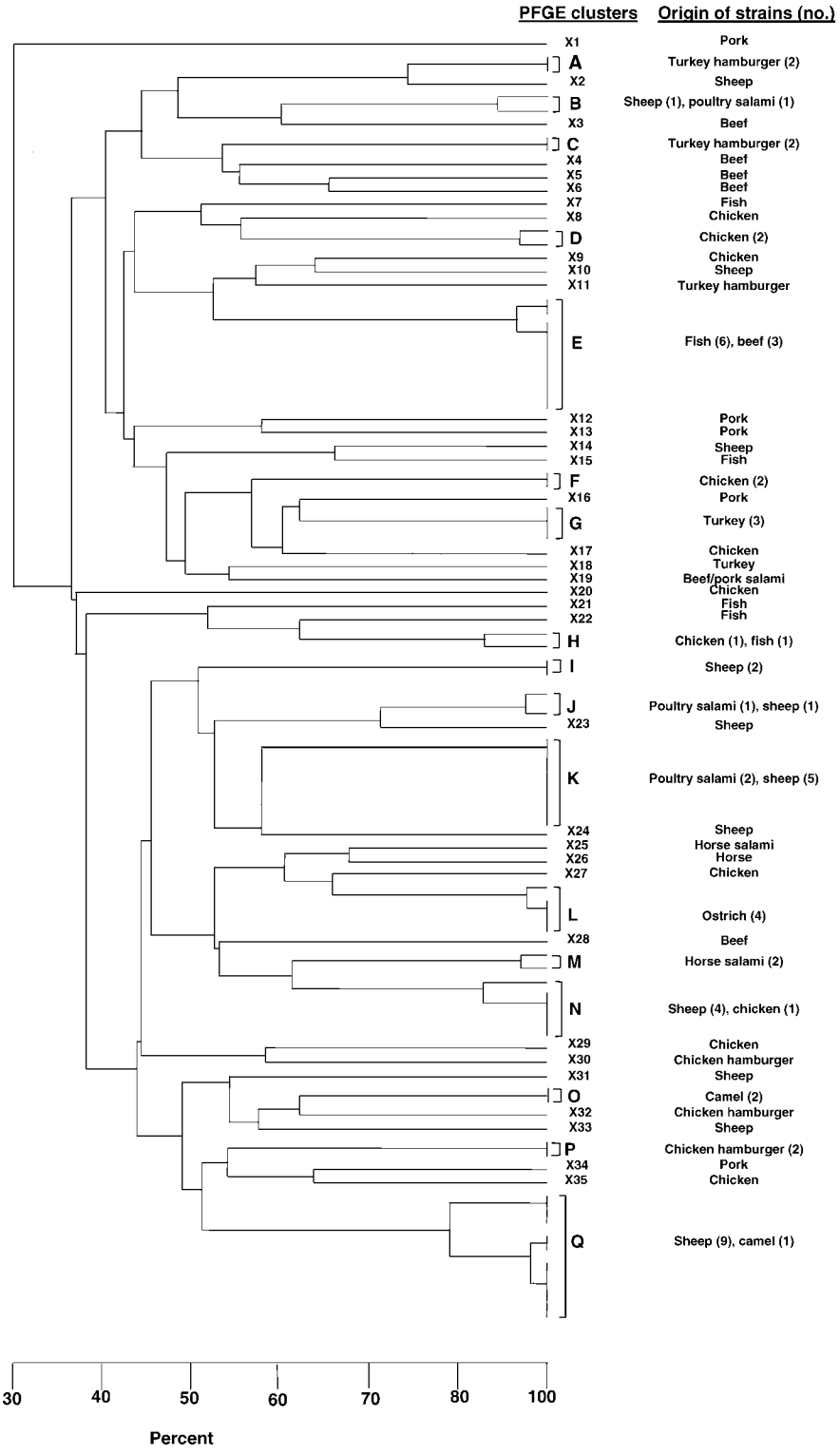

FIGURE 1. *Dendrogram of percent similarities among the PFGE profiles of 98* E. coli *isolates (*Xba*I enzyme failed to produce digestion fragments for three isolates), as calculated using the Dice index. The origin and number of strains for each pattern is indicated.*

in this study; no *E. coli* isolates were obtained from the remaining 13 samples (including 9 samples of elaborated products derived from salami) (Table 1). A total of 98 *E. coli* isolates were obtained. The frequency of *E. coli* recovery from raw food products of animal origin obtained from the supermarket or local butchery (22 of 26 samples, 85%) was higher compared with the level of contamination in elaborated food products such as hamburger or salami (5 of 14 samples, 36%). High contamination by *E. coli* isolates in raw food products of animal origin also has been found in other studies *(7, 24, 35)* and might occur during slaughtering and processing of food products.

Clonal relationships among *E. coli* isolates. The clonal relationships among the 98 *E. coli* isolates were evaluated based on PFGE of *Xba*I-digested genomic DNA. Figure 1 is the dendrogram of all PFGE profiles obtained. It was possible to distinguish 35 unrelated PFGE patterns in 35 unique *E. coli* isolates (patterns X_1 through X_{35}) and 17 unrelated patterns that included clusters of *E. coli* isolates

TABLE 2. *Antimicrobial resistance genes detected among resistant* E. coli *isolates by PCR assay and sequencing*

Antimicrobial	Total no. of resistant *E. coli* isolates	Resistance profile	
		Resistance genes	No. of isolates
Ampicillin	31	bla_{TEM}[a]	16
		bla_{TEM-1b}	15
Tetracycline	41	*tet*(A)	28
		tet(B)	13
Sulphonamide	37	*sul1*	1
		sul2	1
		sul3	1
		sul1, sul2	8
		sul1, sul3	6
		sul2, sul3	13
		sul1, sul2, sul3	7
Trimethoprim-sulfamethoxazole	27	*dfrA1*	12
		dfrA1, dfrA12	1
		dfrA17	1
		dfrA1, dfrA17	1
		dfrA12, dfrA5	2
		dfrA5	10
Streptomycin	27	*aadA1*	25
		aadA5	1
		aadA2	1
Gentamicin	3	*aac(3)*-II	3

[a] The bla_{TEM} gene was detected by PCR assay, but the amplicon was not sequenced.

(patterns A through Q). Three of the *E. coli* isolates were untypeable by PFGE analysis. Two or more different PFGE profiles were identified among the isolates obtained from the same sample in 67% of the *E. coli*–positive samples; in the remaining 33% of these samples, one unique PFGE pattern was identified among the isolates of the same sample.

Antimicrobial resistance phenotypes. The antimicrobial resistance exhibited by our 98 food *E. coli* isolates were as follows: tetracycline (43% of isolates) ampicillin (32%), nalidixic acid (33%), ciprofloxacin (18%), sulphonamides (39%), trimethoprim-sulfamethoxazole (29%), streptomycin (29%), gentamicin (3%), tobramycin (2%), and chloramphenicol (3%). None of the strains were resistant to cefoxitin, cefotaxime, ceftazidime, amikacin, and aztreonam. Similar high percentages of resistance detected for tetracycline, sulphonamides, nalidixic acid, ampicillin, streptomycin, and trimethoprim-sulfamethoxazole (29 to 43%) have been obtained in other countries among *E. coli* isolates from food products or healthy food-producing animals *(9, 15, 17, 19, 24, 35, 36).*

Thirty-one (32%) of the 98 isolates had a multiresistant phenotype that included resistance to at least five different families of antimicrobial agents, and multiresistance was especially high in isolates of chicken and fish origin (60 and 81% of these isolates, respectively). Fifty-four percent of the studied *E. coli* isolates were susceptible to all 15 tested antimicrobial agents, and 80% of isolates recovered from sheep had this phenotype. These results might reflect the different uses of antimicrobial agents in intensive and extensive animal breeding, although more studies would be necessary to test this hypothesis.

Antimicrobial resistance genes. The resistance genes detected among our antimicrobial-resistant *E. coli* isolates are shown in Table 2. Thirty-one ampicillin-resistant isolates were detected in this study, and all of them harbored a bla_{TEM} gene (encoding a TEM beta-lactamase). The bla_{TEM} amplicon was sequenced in 15 of these isolates, and the bla_{TEM-1b} gene was identified in all of them. This gene also was the most prevalent in ampicillin-resistant *E. coli* isolates from foods and food-producing animals in other studies *(3, 9, 23, 35)* and in ampicillin-resistant human isolates *(38).* All of our 41 tetracycline-resistant isolates contained *tet*(A) (68%) or *tet*(B) (32%), and these genes have been reported frequently in human isolates *(38).*

Different combinations of the *sul1, sul2,* and *sul3* genes were found in the 37 sulphonamide-resistant isolates; most of these isolates (92%) harbored more than one *sul* gene, and 19% of the isolates harbored all three *sul* genes. These findings are in agreement with the high prevalence of these genes in *E. coli* isolates from food or animals in other studies *(9, 10, 16, 23, 30).* The *sul3* gene was found in 73% of our sulphonamide-resistant strains recovered

TABLE 3. *Amino acid changes in GyrA and ParC proteins deduced from the sequences of the corresponding genes in the 15 quinolone-resistant* E. coli *isolates*

Origin of isolates	No. of isolates	PFGE patterns	MIC (μg/ml)[a]		Amino acid changes	
			NAL	CIP	GyrA	ParC
Turkey hamburger	1	A	256	0.5	Ser83Leu	Wild
Chicken hamburger	1	X_{30}	512	0.5	Ser83Leu	Wild
Fish	1	E	256	0.5	Ser83Leu	Wild
Chicken	9	D	128	0.5	Ser83Leu	Wild
		N	2,048	16	Ser83Leu + Asp87Gly	Ser80Ile
		X_9, X_{20}, X_{27}, X_{35}, NT	≥2,048	16	Ser83Leu + Asp87Asn	Ser80Ile
		F	>2,048	16	Ser83Leu + Asp87Gly	Glu84Gly
		X_{17}	>2,048	64	Ser83Leu + Asp87Asn	Ser80Ile + Glu84Gly
Turkey	2	X_{18}, G	≥2,048	8	Ser83Leu + Asp87Asn	Ser80Arg
Sheep	1	N	2,048	16	Ser83Leu + Asp87Gly	Ser80Ile

[a] NAL, nalidixic acid; CIP, ciprofloxacin.

TABLE 4. *Analysis of class 1 and class 2 integrons and their resistance cassettes in 15 sulphonamide-resistant* E. coli *isolates with unrelated PFGE patterns*

E. coli isolate	Origin of strain	Antimicrobial resistance phenotype[a]	Class 1 integron			Class 2 integron	
			IntI1	*qacΔE1 sul1*	Gene cassettes in variable region	*IntI2*	Gene cassettes in variable region
EC3a	Chicken hamburger	TET-SXT-SUL- STR[I]	+	−	*dfrA1* + *aadA1*[b]	−	
EC3b	Chicken hamburger	AMP-TET-SXT-SUL-STR[I]	+	+	*dfrA1* + *aadA1*	−	
EC3c	Chicken hamburger	TET-NAL-SXT-SUL- STR	+	+	*dfrA1* + *aadA1*	−	
EC4a	Turkey hamburger	AMP-TET-SXT-SUL-STR	+	+	*dfrA17* + *aadA5*	−	
EC17a	Sheep	AMP-TET-NAL-CIP-SUL-STR	+	+	*aadA1*	−	
EC18b	Chicken	AMP-TET-NAL-CIP-SXT-SUL-STR[I]	+	+	*dfrA1* + *aadA1*[b]	−	
EC19V	Fish	AMP-TET-SXT-SUL-STR-CHL	+	−	*dfrA5*[b]	−	
EC20a	Chicken	AMP-TET-NAL-CIP-SXT-SUL-STR[I]	+	+	*dfrA1* + *aadA1*	−	
EC20b	Chicken	AMP-TET-NAL-CIP-SXT-SUL-STR[I]-CHL	+	+	*dfrA17* + *aadA5*	−	
EC20c	Chicken	TET-NAL-CIP-SXT-SUL-STR	+	+	*dfrA12* + *orfF* + *aadA2*	−	
EC21a	Sheep	AMP-TET-SXT-SUL-STR	+	−	*dfrA5*[b]	−	
EC23a	Chicken	TET-NAL-SXT-SUL-STR	+	+	*dfrA1* + *aadA1*	−	
EC24a	Fish	AMP-TET-SXT-SUL-STR	+	−	*dfrA5*[b]	−	
EC25a	Fish	AMP-TET-NAL-SXT-SUL-STR	+	−	*dfrA5*[b]	−	
EC20d	Chicken	AMP-TET-NAL-CIP-SXT-SUL-STR	−	−		+	*dfrA1* + *sat* + *aadA1*

[a] TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; SUL, sulphonamide; STR, streptomycin; AMP, ampicillin; NAL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol. I, resistance to the drug indicated is in the intermediate category according to CLSI standards for the corresponding strain.

[b] Genes detected by PCR mapping with combinations of primers.

from food products of animal origin (chicken, turkey, sheep, fish, and beef). The *sul3* gene was initially detected in *E. coli* isolates from food animals in Germany, Switzerland, and Spain *(9, 22, 23),* but now it is highly disseminated among *Enterobacteriaceae* of different origins. The association of this gene with insertion sequences and recently with defective class 1 integrons that do not contain the *qacEΔ1-sul1* at their 3′ conserved region *(2, 31)* could explain this prevalence.

The 27 isolates resistant to trimethoprim-sulfamethoxazole presented different types and combinations of *dfrA* genes, some of them identified inside the structure of integrons, with *dfrA1* and *dfrA5* being the most prevalent genes (Table 2). Different types of *aadA* genes (*aadA1, aadA2,* and *aadA5*) also were found in our 27 streptomycin-resistant isolates. Four classes of AAC(3) acetyltransferases (associated with gentamicin resistance in *E. coli*) have been reported. In our three gentamicin-resistant isolates, the *aac(3)*-II gene was identified. The AAC(3)-II and/or AAC(3)-IV enzymes have been previously found in *E. coli* isolates from food-producing animals and humans *(9, 23).*

Mechanisms of quinolone resistance. The mechanisms of quinolone resistance were studied in all the nalidixic acid–resistant isolates that had unrelated PFGE patterns and in those isolates with similar PFGE patterns but different antimicrobial resistance profiles. Fifteen isolates were included in this study, and the results are shown in Table 3. Most of the unrelated isolates were recovered from samples of poultry (13 isolates, 87%). A correlation was found between the type and number of amino acid changes in GyrA and ParC proteins and the MICs of nalidixic acid and ciprofloxacin. Those isolates with nalidixic acid and ciprofloxacin MICs of 128 to 512 and 0.5 μg/ml, respectively, presented one amino acid change in GyrA (Ser83Leu), and the wild sequence in ParC. Those isolates with a MIC of ≥2,048 and 8 to 16 μg/ml, respectively, presented two amino acid changes in GyrA (Ser83Leu + Asp87Asn, or Ser83Leu + Asp87Gly) and one amino acid change in ParC (Ser80Arg, Ser80Ile, or Glu84Gly). The highest MICs of nalidixic acid and ciprofloxacin (≥2,048 and 64 μg/ml, respectively) were associated with the presence of two amino acid changes in GyrA (Ser83Leu + Asp87Asn) and two others in ParC (Ser80Ile + Glu84Gly) (Table 3). This observation has been previously reported in human and animal *E. coli* isolates *(25, 36).*

Characterization of integrons in sulphonamide-resistant isolates. Integrons were detected and characterized in 15 of our 98 *E. coli* isolates. These isolates were resistant to sulphonamides and to a large group of antimicrobial agents of different families, and they had unrelated PFGE patterns, with the exception of two isolates that had the same PFGE pattern but different antimicrobial resistance profiles. Table 4 shows the characteristics of these 15 isolates, the type of integrons detected, and the antimicrobial resistance gene cassettes.

The class 1 integrase gene, *intI1,* was detected in 14 of the 15 studied isolates. Five *intI1*-positive isolates lacked the *qacEΔ1-sul1* region in the 3′ conserved region of class

1 integrons and four of them contained the *sul3* gene. The existence of these defective integrons lacking the *qacE*Δ*1-sul1* region has been recently reported *(2, 31, 38),* and further studies will be carried out to determine the complete structure of these defective integrons. The variable region of the 14 *intI1*-positive isolates ranged in size from 1,000 to 2,000 bp, and the following gene cassette arrangements were identified by sequencing: *dfrA1* + *aadA1* (6 isolates), *dfrA5* (4 isolates), *dfrA17* + *aadA5* (2 isolates), *aadA1* (1 isolate), and *dfrA12* + *orfF* + *aadA2* (1 isolate). One isolate harbored a class 2 integron that included the *dfrA1* + *sat* + *aadA1* gene cassette arrangement. Ten (67%) of the 15 isolates that harbored integrons were recovered from samples of poultry. The detection of various sizes of integrons and arrangements of gene cassettes involved in resistance to streptomycin (*aadA1, aadA2,* and *aadA5)* and trimethoprim (*dfrA1, dfrA17, dfrA5,* and *dfrA12)* in the *E. coli* isolates of this study also was previously reported in human *E. coli* isolates and food isolates *(9, 15, 23, 28, 30).* Most of our isolates that were positive for class 1 integrons harbored the *tet*(A) gene, and others have suggested that the *tet(*A) gene and class 1 integrons are often located on the same conjugative plasmid *(30).*

Raw food of animal origin can contain *E. coli* isolates that are resistant to multiple antimicrobials and that harbor a wide variety of antimicrobial resistance genes and genetic structures for acquisition and accumulation of these genes, as is the case of integrons. The application of good hygiene practices throughout the food chain and the prudent use of antimicrobial agents in food-producing animals are important issues that should be addressed at the global level. Food safety is a public health priority that requires a global approach from production to consumption. Monitoring of antimicrobial resistance in food bacteria of human interest should continue to track the evolution of these bacteria and to assess the possible risks for human health.

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