Fluoroquinolone resistance and molecular characterization of *gyrA* and *parC* quinolone resistance-determining regions in *Escherichia coli* isolated from poultry

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ABSTRACT *Escherichia coli* are a common inhabitant of the gastrointestinal tract of mammals and birds; nevertheless, they may be associated with a variety of severe and invasive infections. Whereas fluoroquinolones (FQ) have been banned in the United States for use in poultry production, the use of these antimicrobials in poultry husbandry is still possible in the European Union, although with some restrictions. The aim of this study was to investigate the FQ resistance of 235 *E. coli* isolates recovered from chickens and turkeys. Minimum inhibitory concentrations were determined by a microdilution method, whereas mutations in the quinolone resistance-determining regions of the target genes, *gyrA* and *parC*, were detected by a PCR-based method. High resistance rates (>60%) were observed for nalidixic acid, flumequine, and difloxacin, whereas resistance to ciprofloxacin, danofloxacin, enrofloxacin, marbofloxacin, and sarafloxacin was less frequently reported (<40%). Sixty-four isolates (27.2%) showed full susceptibility toward the tested FQ, but 57 isolates (24.2%) were resistant to all tested FQ. The remaining 114 *E. coli* isolates (48.5%) were grouped in 5 different resistance patterns. Isolates resistant only to flumequine or nalidixic acid or both possessed 1 *gyrA* mutation, whereas isolates with further resistance to enrofloxacin, difloxacin, danofloxacin, and sarafloxacin had in addition 1 or 2 *parC* substitutions. Two *gyrA* mutations coupled with 1 substitution in *parC* were detected in isolates resistant to all tested FQ. The number of mutations and their correlation with the in vitro activity of FQ reflected the currently accepted model, according to which a single *gyrA* substitution is associated with resistance or decreased susceptibility to older quinolones, whereas further *gyrA* or *parC* substitutions are needed for a higher level of resistance.

Key words: *Escherichia coli*, poultry, antimicrobial resistance, fluoroquinolone

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INTRODUCTION

Escherichia coli are generally considered as a common inhabitant of the gastrointestinal tract of mammals and birds; nevertheless, strains possessing specific virulence factors may be associated with a variety of severe and invasive infections (Kaper et al., 2004). Avian pathogenic *E. coli* is responsible for different local and systemic diseases of poultry, such as colibacillosis, a severe systemic disease that is considered a major cause of morbidity and mortality in the poultry industry worldwide (Ahmed et al., 2013). Moreover, the avian intestinal environment has been considered as a reservoir of potentially zoonotic *E. coli*, with the subsequent possible contamination of poultry products with such bacteria during slaughter (Wasteson, 2001).

Treatment strategies include attempts to control predisposing infections or environmental factors and early use of antibacterials indicated by susceptibility tests. Potentiated sulfonamides are considered as first choice antimicrobial agents for the treatment of avian colibacillosis, whereas tetracyclines and aminopenicillins (ampicillin, amoxicillin) should be used as second choice antimicrobials (Löhren et al., 2008). Fluoroquinolones (**FQ**) have been widely used in poultry since their introduction in the early 1990s (Prescott and Baggot, 1993). The advantage of oral administration, bactericidal activity at low tissue concentrations and high potency against many gram-negative microorganisms,

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Table 1. Fluoroquinolone (FQ) resistance rates and minimum inhibitory concentrations (MIC) of *Escherichia coli* isolated from poultry in Europe, United States (US), and Asian countries

FQ	Resistant isolates (%)	MIC range	Country (source)	Reference
Ciprofloxacin	75.2	nr[1]	China (chickens)	Liu et al., 2012
	73	>16[2]	China (chickens)	Yang et al., 2004
	60.2	3–≥32	South Korea (chickens)	Lee et al., 2005
	50	nr	UK (turkeys)	Gosling et al., 2012
	43	nr	Bolivia (chickens)	Riccobono et al., 2012
	5	0.12–8	US (chickens)	Zhao et al., 2005
	nr	0.03–64	Spain (broilers)	Sáenz et al., 2003
Danofloxacin	23.6	0.25[2]	Japan (chickens)	Ozawa et al., 2010
	16	0.25–16	US (chickens)	Zhao et al., 2005
Difloxacin	91	>16[2]	China (chickens)	Yang et al., 2004
	57	1–>16	US (chickens)	Zhao et al., 2005
Enrofloxacin	90	>16[2]	China (chickens)	Yang et al., 2004
	81	nr	China (chickens)	Liu et al., 2012
	73.4	2–≥32	South Korea (chickens)	Lee et al., 2005
	40	0.015–8	France (turkeys)	Giraud et al., 2001
	24.4	0.25[2]	Japan (chickens)	Ozawa et al., 2010
	16	0.25–16	US (chickens)	Zhao et al., 2005
Flumequine	48	0.5–256	France (turkeys)	Giraud et al., 2001
Nalidixic acid	100	>256[2]	China (chickens)	Yang et al., 2004
	100	64–>256	US (chickens)	Zhao et al., 2005
	97.4	nr	China (chickens)	Liu et al., 2012
	81	nr	Bolivia (chickens)	Riccobono et al., 2012
	49	4–>1,024	France (turkeys)	Giraud et al., 2001
	nr	32–>256	Spain (broilers)	Sáenz et al., 2003
Norfloxacin	60.2	16–≥32	South Korea (chickens)	Lee et al., 2005
	22.8	0.5[2]	Japan (chickens)	Ozawa et al., 2010
Sarafloxacin	100	>16[2]	China (chickens)	Yang et al., 2004

[1]nr = not reported.

[2]Mean MIC value.

made FQ potentially useful in the treatment of colibacillosis and other infections caused by *E. coli* in chickens and turkeys. Nevertheless, the widespread use of FQ in the poultry industry, and the subsequent selective pressure on bacteria of animal origin, contributed to the acquisition of resistance toward this antimicrobial class and reduction in their therapeutic efficacy (Harada and Asai, 2010).

A growing number of studies have reported a high level of FQ-resistant *E. coli* isolated from poultry, mostly to nalidixic acid and ciprofloxacin, with minimum inhibitory concentration (**MIC**) values ranging from 4 to >1,024 and from 0.003 to ≥32 μg/mL, respectively (Table 1). Moreover, isolates displaying resistance to nalidixic acid are often co-resistant to difloxacin, enrofloxacin, and norfloxacin. Nevertheless, the use of FQ, such as enrofloxacin, danofloxacin, and difloxacin, is often inevitable due to the increasing level of resistance to first or second choice antimicrobials observed in recent years (Haritova et al., 2006; Löhren et al., 2008; Furtula et al., 2010; de Jong et al., 2012). To face these problems, international bodies and health authorities have concerns on development of FQ resistance in human and animal pathogens, including *E. coli*. The World Health Organization has classified these antimicrobials as critically important in human medicine based on the evidence that antimicrobial resistance in *E. coli* infections in humans may derive from farm antibiotic use and that resistant *E. coli* can spread from farm animals to humans (WHO, 2011).

Improved management of the use of FQ in food animals, particularly reducing those critically important for human medicine, is an important step toward preserving the benefits of these antimicrobials. In 2005, the US Food and Drug Administration banned the use of FQ in chickens and turkeys because of concerns about increasing resistance in *E. coli* and other zoonotic bacteria (Goetting et al., 2011). In the European Union, a reflection paper on the prudent use of FQ in food producing animals was adopted in 2006 by the European Medicines Agency (EMEA, 2006) to critically review its effect on the development of FQ resistance in zoonotic bacteria and the potential effect on human and animal health. On the basis of the European Union-published directives, FQ have been approved for the treatment of bacterial infections in poultry. As a consequence, FQ continue to be used in the poultry industry in Europe, thereby contributing to maintain a relatively high resistance level toward this antimicrobial class (EMEA, 2012).

The most frequent mechanism of FQ resistance in *E. coli* is represented by alterations occurring in genes encoding for DNA gyrase and topoisomerase IV, the FQ primary and secondary target enzymes in gram-negative bacteria, respectively, both playing vital roles in DNA replication and chromosome segregation (Fàbrega et al., 2008). Alterations involve mainly chromosomal mutations located in the quinolone resistance-determining regions (**QRDR**) of the *gyrA* and *gyrB* genes, which encode for the 2 DNA gyrase subunits,

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and its homologous region of the *parC* and *parE* genes, encoding for the 2 topoisomerase IV subunits. Other mechanisms affecting the bacterial accumulation of FQ, such as efflux pump systems activity or modifications of porins, as well as target protection and drug enzymatic modification, can contribute to enhance the degree of resistance in *E. coli* (Fàbrega et al., 2008). The aim of the present study was to investigate the phenotypic resistance toward FQ and the presence of QRDR mutations in the *gyrA* and *parC* genes of *E. coli* isolates isolated from poultry samples from 2010 to 2012 in Italy.

MATERIALS AND METHODS

Bacterial Isolates

A total of 235 *E. coli* isolates recovered from fecal samples and viscera (brain, pericardial sac, liver, lungs, and joints) from chickens and turkeys were included in this study. The isolates were collected from 2010 to 2012 from different colibacillosis outbreaks that occurred in 80 industrial poultry farms located in a densely populated poultry area of northern Italy. Poultry affected by clinical disease with suspected colisepticemia (at least 3 wk of age) were euthanized and necropsy was carried out immediately after to avoid postmortem contamination of the carcass. No more than 1 isolate of *E. coli* from the same farm per year was included in the study. The animals had not been treated with antimicrobial agents in the 3 wk before sample collection. Samples were processed for bacterial isolation on sheep blood agar (Oxoid, Milano, Italy) and Eosin Methylene Blue agar (Oxoid) and incubated aerobically at 37°C for 24 h. Presumptive *E. coli* isolates were confirmed using the RapID E 20 kit (Biomérieux, Mercy l'Etoile, France) following the manufacturer's instructions. All isolates were immediately subcultured on sheep blood agar and subjected to antimicrobial susceptibility testing.

Antimicrobial Susceptibility Testing

The MIC of ciprofloxacin (**CIP**), danofloxacin (**DAN**), difloxacin (**DIF**), enrofloxacin (**ENR**), flumequine (**FLU**), marbofloxacin (**MAR**), nalidixic acid (**NAL**), and sarafloxacin (**SAR**) were determined by the broth microdilution method with Mueller-Hinton broth (Oxoid) in a 96-well plate (Steroglass, Perugia, Italy), following the guidelines proposed by the Committee for Antibiogram of the French Society of Microbiology (for ENR, FLU, MAR, NAL, and CIP) and those proposed by the Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (for DAN, DIF, and SAR; NCCLS, 2003a,b; CLSI, 2008; CASFM, 2011). Antimicrobials were purchased as powders suitable for susceptibility testing (Sigma-Aldrich, Milano, Italy) and stock solutions were prepared following the manufacturer's instructions, divided into aliquots of approximately 1 mL and kept at −20°C until needed. The tests were done with freshly prepared dilutions of the stock solutions. For each FQ, 16 concentrations in 2-fold dilution series, ranging from 0.0078 to 256 μg/mL, were tested. The inoculum was prepared by suspending bacterial colonies from an overnight sheep blood agar culture in sterile 0.9% saline and adjusting the turbidity to a 0.5 McFarland standard (ca. 10^8 cfu/mL). The suspension was further diluted to provide a final inoculum of 5×10^4 cfu/well. All plates were incubated aerobically at 37°C for 16 to 18 h. Each test was performed in triplicate. Regular quality assurance was performed by using the American Type Culture Collection reference strain *E. coli* ATCC 25922 (Oxoid). The MIC of each FQ was defined as the lowest concentration of antimicrobial that completely inhibits growth of the tested isolate in the microdilution wells as detected by the unaided eye (absence of turbidity) compared with the positive control (antibiotic-free well).

Minimum inhibitory concentration of FQ at which 50% of the isolates were inhibited ($\mathbf{MIC_{50}}$) were also calculated. Isolates were further classified as resistant or susceptible to the tested FQ according to breakpoint proposed by the Committee for Antibiogram of the French Society of Microbiology for *Enterobacteriaceae* of animal origin (for ENR, FLU, MAR, NAL) and human origin (CIP; CASFM, 2011); whereas for DAN, DIF, and SAR, breakpoints adopted by the Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards for bacteria isolated from animals were applied (Table 2; NCCLS, 2003a,b; CLSI, 2008). Isolates which showed intermediate susceptibility to FQ were included with the resistant ones.

Amplification and DNA Sequencing of gyrA and parC QRDR

Four to 5 bacterial colonies, depending on their size, of a sheep blood agar overnight culture were suspended in 900 μL of 20 m*M* Tris-HCl buffer (pH 7.6), incubated at room temperature for 10 min and centrifuged for 2 min at 9,000 × *g* and 4°C. Pellets were then resuspended in 600 μL of lysis buffer (10 m*M* Tris-HCl,

Table 2. Fluoroquinolone (FQ) tested and minimum inhibitory concentrations (MIC) breakpoint

	MIC breakpoint[1]		
FQ	R	I	S
Ciprofloxacin	>1	1	≤0.5
Danofloxacin	>0.25	—	≤0.25
Difloxacin	>2	1–2	≤0.5
Enrofloxacin	>2	1–2	≤0.5
Flumequine	>8	8	≤4
Marbofloxacin	>2	2	≤1
Nalidixic acid	>16	16	≤8
Sarafloxacin	≥0.25	0.125	≤0.06

[1]R = resistant; I = intermediate; S = susceptible.

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400 m*M* NaCl, 2 m*M* sodium EDTA, pH 7.6) containing 50 μL of 10% SDS and 6 μL of proteinase K (100 μg/mL), vortexed, and incubated at 55°C. After 4 h, samples were cooled, added to 3 μL of RNAase (4 mg/mL), incubated at 37°C for 1 h, supplemented with 200 μL of potassium acetate, vortexed, and centrifuged for 3 min at 9,000 × *g* and 4°C. Finally, supernatants were removed and DNA was purified by addition of isopropanol-alcohol and precipitation with ethanol. The DNA was resuspended in 50 μL of sterile water and stored at 4°C until use.

A 347-bp fragment (from nucleotides 108–454) containing the QRDR of *gyrA* was amplified using primers P1F (5′-TGTCCGAGATGGCCTGAAGC-3′) and P2R (5′-TACCGTCATAGTTATCCACG-3′; Griggs et al., 1996). A 964-bp fragment (from nucleotides 17–981) containing the QRDR region of *parC* was amplified with primers ParCF43 (5′-AGCGCCTTGCGTACATGAAT-3′) and ParCR981 (5′-GTGGTAGCGAAGAGGTGGTT-3′; Komp Lindgren et al., 2003). Amplification was performed in a final volume of 50 μL containing each primer at a concentration of 0.2 μ*M*, 1 × PCR buffer, 200 μ*M* (each) deoxynucleotide phosphate, 3 m*M* $MgCl_2$, 2.5 U of Taq polymerase (Promega, Madison, WI), and approximately 100 ng of chromosomal DNA. The PCR reactions were performed using an iCycler iQ Real-Time PCR system (Bio-Rad, Hercules, CA) with an initial denaturing cycle at 94°C for 5 min followed by 33 cycles of 94°C for 1 min, 54.5°C for 1 min (*gyrA*) or 54°C for 1 min (*parC*), and 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplified PCR products for *gyrA* and *parC* were sequenced on both strands by using the BigDye Terminator Cycle Sequencing Kit v3.1 in the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Editing of chromatograms and assembly of nucleotide sequences were performed by using the software ChromasPro v. 1.42 (Technelysium Pty Ltd., Tewantin, Australia). The resulting DNA sequences were compared with the *E. coli gyrA* and *parC* genes from GenBank at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/blast).

RESULTS

FQ Resistance Phenotypes of E. coli Isolates

The MIC distributions, MIC_{50} values, and FQ resistance rates of the 235 *E. coli* isolates are shown in Table 3. The large majority of isolates showed high resistance rates to NAL (72.8%, n = 171), FLU (70.2%, n = 165), and DIF (65.1%, n = 153). Resistance was less frequently observed to ENR (38.7%, n = 91), DAN (26.8%, n = 63), and SAR (26.8%, n = 63). The lowest resistance rates were observed for CIP (24.2%, n = 57) and MAR (24.2%, n = 57). Sixty-four isolates (27.2%) showed full susceptibility to all tested FQ, whereas 57

Table 3. Minimum inhibitory concentration (MIC) distributions and fluoroquinolone (FQ) resistance rates of 235 *Escherichia coli* isolated from poultry

FQ[1]	Number of isolates with MIC[2] (μg/mL)													MIC_{50}[3] (μg/mL)	Resistance rates[4] (%)
	≤0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256		
NAL								2	14	36	12			**2**	**14**	**155**	≥256	72.8
FLU		1			1	7	39	12	5	5	**4**	**75**	**17**	**4**	**5**	**60**	16	70.2
DIF				4	26	25	27		**4**	**38**	**47**	**8**	**6**	**4**	**9**	**37**	4	65.1
ENR	10	22	16	10	5	19	62	**26**	**2**	**12**	4	**11**	**20**	**7**	**5**	**4**	0.5	38.7
DAN	44	14	2	8	30	74	**1**	**3**	**4**	**6**	**18**	**20**	**3**	**4**	**2**	**2**	0.25	26.8
SAR	46	12	21	93	6			**1**	**4**	**9**	**4**	**24**	**13**			**2**	0.06	26.8
CIP	57	6	13	44	43	10	5	**6**	**8**	**22**	**11**	**4**	**3**	**3**			0.06	24.2
MAR	5	39	15	1	4	30	63	21	**7**	**6**	**24**	**12**	**6**		**2**		0.5	24.2

[1]NAL = nalidixic acid; FLU = flumequine; DIF = difloxacin; ENR = enrofloxacin; DAN = danofloxacin; SAR = sarafloxacin; CIP = ciprofloxacin, MAR = marbofloxacin.
[2]Bold numbers indicate resistant isolates.
[3]MIC_{50} = at given concentration of antimicrobials, 50% of tested isolates were inhibited.
[4]Isolates with intermediate susceptibility were grouped with the resistant ones.

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isolates (24.2%) were resistant to all tested FQ (Table 4). The remaining *E. coli* isolates were grouped in 5 different resistance patterns: 62 isolates (26.4%) showed resistance to DIF, FLU and NAL; 28 isolates (11.9%) showed a DIF-ENR-FLU-NAL resistance pattern; 12 (5.1%) were resistant to FLU-NAL; 6 isolates (2.6%) showed a DAN-DIF-ENR-FLU-NAL-SAR resistance; and 6 isolates (2.6%) were only NAL-resistant.

Analysis of gyrA and parC Mutations

The amino acid changes in the QRDR of *gyrA* and *parC* and the FQ MIC_{50} values of *E. coli* isolates are reported in Table 5. Isolates that were resistant only to NAL or to NAL and FLU possessed a *gyrA* mutation at codon 83, substituting a serine with a leucine (Ser83Leu). Isolates with further resistance to DIF, or to DIF and ENR, had, in addition to the *gyrA* Ser-83Leu mutation, 1 or 2 *parC* substitutions, replacing an alanine with a valine at codon 108 (Ala108Val) or an asparagine with a serine at codon 91 (Asn91Ser). Similarly, isolates with the DAN-DIF-ENR-FLU-NAL-SAR resistance pattern had, in addition to 1 *gyrA* mutation at codon 83, 1 or 2 *parC* substitutions, replacing a serine with an arginine (Ser80Arg) or an aspartic acid (Ser80Asp) at codon 80 or an asparagine with a serine at codon 91 (Asn91Ser). Two *gyrA* mutations at codons 83 (Ser83Leu) and 87 (Asp87Asn) coupled with a serine to isoleucine substitution in *parC* at codon 80 (Ser80Ile) were detected in isolates resistant to all tested FQ.

DISCUSSION

Escherichia coli isolated from poultry of different geographic origins have been frequently reported to be resistant to several antimicrobial classes, including β-lactams, tetracyclines, potentiated sulfonamides, and aminoglycosides (Vandemaele et al., 2002; Yang et al., 2004; Zhao et al., 2005; Egea et al., 2012; Zhao et al., 2012). Owing to their broad spectrum of activity against a wide range of bacteria and due to their favorable pharmacokinetic profile, FQ are frequently used in the treatment of colibacillosis and other bacterial infections in chicken and turkey. However, the prevalence and dissemination of resistance toward this class of antimicrobial agents in avian *E. coli* increased significantly in recent years, which is an alarming public-health concern, especially considering that FQ-resistant *E. coli* often exhibits a multidrug-resistant phenotype (Ozawa et al., 2010; Gosling et al., 2012; Liu et al., 2012; Riccobono et al., 2012).

Phenotypical FQ Resistance

Data from the present study are consistent with those of recent reports, with high resistance rates observed toward older quinolones, such as NAL and FLU, as well as DIF, which is currently approved for use in poultry husbandry in many European countries (Andreu et al., 2007). Lower resistance rates, similar to those recently reported, were observed for second and third generation FQ DAN, ENR, and SAR (26.8%), and for CIP and MAR (24.2%), whose MIC_{50} values ranged between 0.06 and 0.5 μg/mL (Gosling et al., 2012; Ozawa et al., 2010; Russo et al., 2012).

Resistance to Older Quinolones: Single gyrA Mutation

Bacterial resistance to FQ develops mainly by accumulation of target enzymes point mutations. Topoisomerase IV tends to be the primary target of FQ in gram-positive bacteria; whereas in gram-negative bacteria, including *E. coli*, DNA gyrase is the primary target and topoisomerase IV is less sensitive to FQ action, thus representing a secondary target compared with DNA gyrase (Zhao et al., 2005; Intorre et al., 2007). Mutation replacing a serine with a leucine at codon 83 (Ser-83Leu) is the most frequently identified in *E. coli* from chicken and turkey with resistance to older quinolones (nalidixic acid, flumequine) and reduced susceptibility to second and third generation FQ, such as ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin, and gatifloxacin (Giraud et al., 2001; Sáenz et al., 2003; Yang et al., 2004; Lee et al., 2005). In our study, *gyrA* analysis among avian *E. coli* isolates confirmed Ser83Leu as a

Table 4. Phenotypical fluoroquinolone (FQ) resistance patterns of 235 *Escherichia coli* isolated from poultry

FQ resistance (n)	FQ resistance pattern[1]	Number of isolates[2] (%)
0	No resistance	64 (27.2)
1	NAL	6 (2.6)
2	FLU-NAL	12 (5.1)
3	DIF-FLU-NAL	62 (26.4)
4	DIF-ENR-FLU-NAL	28 (11.9)
6	DAN-DIF-ENR-FLU-NAL-SAR	6 (2.6)
8	CIP-DAN-DIF-ENR-FLU-MAR-NAL-SAR	57 (24.2)

[1]CIP = ciprofloxacin; DAN = danofloxacin; DIF = difloxacin; ENR = enrofloxacin; FLU = flumequine; MAR = marbofloxacin; NAL = nalidixic acid; SAR = sarafloxacin.

[2]Isolates with intermediate susceptibility were grouped with the resistant ones.

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hot spot mutation, being present in 37 out of 39 (95%) of the sequenced isolates with *gyrA* alterations. Moreover, isolates showing only the *gyrA* Ser83Leu substitution resulted resistant to NAL and FLU but they were still susceptible to CIP, DAN, DIF, ENR, MAR, and SAR, although with an average 3.6 doubling dilution difference in MIC_{50} values compared with wild-type isolates.

Low Level of Resistance to FQ: 1 gyrA or 1 or 2 parC Mutations

Once a first-step mutation has reduced the sensitivity of DNA gyrase, alteration of secondary target topoisomerase IV can further reduce the activity of FQ, extending resistance to enrofloxacin, ciprofloxacin, levofloxacin, and gatifloxacin. Isolates with single *gyrA* mutation coupled with mutations in *parC* have been frequently observed in poultry isolates of *E. coli*, mostly at codon 80, resulting in amino acid substitutions of a serine with an arginine (Ser80Arg) or an isoleucine (Ser80Ile) (Giraud et al., 2001; Sáenz et al., 2003; Yang et al., 2004; Lee et al., 2005; Liu et al., 2012). As previously reported, *parC* mutations are usually associated with a variable level of FQ resistance. Our results are consistent with those of previous reports because *E. coli* isolates with 1 *gyrA* mutation coupled with 1 or 2 mutations in *parC* were resistant to FLU, NAL, and DIF, as well as ENR, DAN, and SAR. A major finding of the present study was the observation that *parC* substitutions at codon 80 replacing a serine with an arginine (Ser80Arg) or an aspartic acid (Ser80Asp), compared with substitution at codon 108 replacing an alanine with a valine (Ala108Val), were generally associated with higher MIC_{50} values and resistance level. The Ala108Val mutation resulted in resistance only to FLU, NAL, and DIF. On the contrary, Ser80Arg or Ser80Asp substitutions led to a marked increase in MIC_{50} values for DAN, DIF, ENR, FLU, MAR, and SAR compared with *parC* wild-type isolates. To the author's knowledge, the *parC* mutation Asn91Ser has not been reported previously in avian *E. coli*, although the presence of this substitution in isolates that already had one mutation in *parC* has not determined a higher level of resistance, both in terms of MIC_{50} values and in the number of FQ toward which the isolates were resistant.

High Level of Resistance to FQ: 2 gyrA or 1 or 2 parC Mutations

A single mutation in *gyrA*, coupled with one or more *parC* mutations, is sufficient to confer a moderate level of resistance to FQ widely used in poultry husbandry, such as nalidixic acid, danofloxacin, difloxacin, and enrofloxacin, whereas recent reports have shown that additional alterations of DNA gyrase are necessary to reach a high level of resistance. The relationship between a high level of FQ resistance and a second *gyrA*

Table 5. Mutations in *gyrA* and *parC* and corresponding fluoroquinolone (FQ) resistance patterns in *Escherichia coli* isolated from poultry

Amino acid substitution(s)[1]		MIC_{50}[2,3] (μg/mL; doubling dilution difference compared with *gyrA*-wild type isolates)								
gyrA	*parC*	CIP	DAN	DIF	ENR	FLU	MAR	NAL	SAR	Resistance pattern
Wild-type	Wild-type	0.008	0.008	0.25	0.016	0.5	0.016	4	0.008	No resistance
Ser83Leu	Wild-type	0.06 (3)	0.125 (4)	0.5 (1)	0.5 (5)	4 (3)	0.5 (5)	128 (5)	0.06 (3)	NAL
Ser83Leu	Wild-type	0.06 (3)	0.125 (4)	0.5 (1)	0.25 (4)	16 (5)	0.25 (4)	256 (6)	0.06 (3)	FLU-NAL
Ser83Leu	Ala108Val	0.125 (4)	0.25 (5)	8 (5)	1 (6)	16 (5)	0.5 (5)	>256 (>6)	0.06 (3)	DIF-ENR-FLU-NAL
Ser83Leu	Ala108Val	0.06 (3)	0.25 (5)	4 (4)	0.5 (5)	16 (5)	0.5 (5)	256 (6)	0.06 (3)	DIF-FLU-NAL
Ser83Leu	Ala108Val, Asn91Ser	0.06 (3)	0.25 (5)	4 (4)	0.5 (5)	16 (5)	0.5 (5)	256 (6)	0.06 (3)	DIF-FLU-NAL
Ser83Leu	Ser80Arg	0.5 (6)	1 (7)	32 (7)	4 (8)	256 (9)	1 (6)	>256 (>6)	4 (9)	DAN-DIF-ENR-FLU-NAL-SAR
Ser83Val	Ser80Asp	0.5 (6)	1 (7)	32 (7)	4 (8)	256 (9)	1 (6)	>256 (>6)	4 (9)	DAN-DIF-ENR-FLU-NAL-SAR
Ser83Leu	Ser80Arg, Asn91Ser	0.25 (5)	2 (8)	32 (7)	4 (8)	>256 (>9)	1 (6)	>256 (>6)	2 (8)	DAN-DIF-ENR-FLU-NAL-SAR
Ser83Leu, Asp87Asn	Ser80Ile	4 (9)	16 (11)	256 (10)	32 (11)	>256 (>9)	8 (9)	>256 (>6)	16 (11)	CIP-DAN-DIF-ENR-FLU-MAR-NAL-SAR

[1]Substituted amino acids and position number (e.g., Ser83Leu indicates substitution of a serine with a leucine at position 83).
[2]MIC_{50} = at given concentration of antimicrobials, 50% of tested isolates were inhibited.
[3]CIP = ciprofloxacin; DAN = danofloxacin; DIF = difloxacin; ENR = enrofloxacin; FLU = flumequine; MAR = marbofloxacin; NAL = nalidixic acid; SAR = sarafloxacin.

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mutation at codon 87, replacing an aspartic acid with an asparagine (Asp87Asn) or a tyrosine (Asp87Tyr), has been well characterized in *E. coli* isolated from poultry (Sáenz et al., 2003; Yang et al., 2004; Zhao et al., 2005; Gosling et al., 2012; Zhao et al., 2012). Accordingly, in the present study, 2 *gyrA* mutations (Ser-83Leu and Asp87Asn) coupled with a Ser80Ile substitution in *parC* were observed only in isolates that were fully resistant to all tested FQ, resulting in an average 10 doubling dilution difference with wild-type isolates.

Conclusions

In conclusion, the high prevalence of FQ-resistant *E. coli* of poultry origin found in our study, mostly toward nalidixic acid, flumequine, and difloxacin, stresses the importance of prudent use of these antimicrobial agents and the need for further surveillance programs. Whereas FQ have been banned in the United States in chicken and turkey, these antimicrobials are currently approved in poultry production in European countries. Despite control strategies based on antimicrobial restriction policies that have been adopted by several health authorities, FQ continue to be widely used in poultry production in European countries, possibly contributing to a high level of resistance. Therefore, the presence of FQ-resistant isolates should be routinely screened to preserve the efficacy of these important antimicrobial agents. The present study confirms alterations of target enzymes DNA gyrase and topoisomerase IV as a determining factor for the development of FQ resistance in *E. coli*. The correlation between the number and type of amino acid substitutions and the in vitro activity of FQ showed that multiple *gyrA* and *parC* mutations are necessary for a high level of resistance, often reflecting in different FQ resistance patterns depending on the affected codon.

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