

## Resistance to Fluoroquinolones in *Escherichia coli* Isolated from Poultry

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**Quinolone-resistant *Escherichia coli* strains were isolated from poultry clinical samples in Saudi Arabia. The poultry flocks had been treated with oxolinic acid or flumequine prophylaxis. The measure of the uptake of fluoroquinolones showed that none of the strains had a reduced accumulation of quinolones. The result of complementation with the wild-type *E. coli gyrA* gene, which restored fluoroquinolone susceptibility, and the isolation of DNA gyrase from six isolates indicated that the resistant strains had an altered DNA gyrase. The minimum effective dose of ciprofloxacin for inhibition of supercoiling catalyzed by the isolated gyrases varied from 0.085 µg/ml for a susceptible isolate (MIC < 4 µg/ml) up to 96 µg/ml for the more resistant one (strain 215, MIC > 64 µg/ml). For the same two isolates, the minimum effective doses of sparfloxacin varied from 0.17 up to 380 µg/ml. The in vitro selection of spontaneous single-step fluoroquinolone-resistant mutants using ciprofloxacin suggested that the more resistant mutants are likely the result of several mutations. These results also show that, as in human medicine, cross-resistance between older quinolones and fluoroquinolones can exist in veterinary isolates and reiterate the need for the prudent use of these drugs.**

The fluoroquinolones are antibacterial agents that were introduced in the mid-1980s in human medicine, and the emergence of fluoroquinolone resistance has been most commonly observed in bacteria other than *Escherichia coli* (20–23, 26, 29–32). Thus, in uncomplicated urinary tract infections caused by *E. coli*, the current incidence of resistance is not usually greater than 3%. Only a few reports about quinolone resistance in veterinary practice have recently been published, concerning *E. coli* (5), *Campylobacter jejuni* (11), *Salmonella* spp. (5, 12, 25), and *Aeromonas salmonicida* (20).

Flumequine, the first fluoroquinolone, was synthesized in 1970. It was introduced into therapy in 1978 and is mainly used in uncomplicated urinary tract infections. It showed improved antibacterial activity relative to those of nalidixic and oxolinic acids (26, 29) (MICs for most gram-negative clinical isolates except *Pseudomonas aeruginosa*, <4 µg/ml; MIC for *Staphylococcus aureus*, ~4 µg/ml). The advantages of flumequine over nonfluorinated quinolones prompted its use in veterinary situations, in which it was the only fluorinated quinolone used until the early 1990s.

The present study bears on 15 quinolone-resistant strains of *E. coli* isolated in Saudi Arabia from poultry clinical material originating from flocks having undergone oxolinic acid or flumequine treatment. Flumequine and the nonfluorinated quinolone oxolinic acid were introduced in 1987 for veterinary use in Saudi Arabia. In the following year, diagnostic laboratories observed an increase in the proportion of quinolone-resistant isolates from poultry clinical samples (20a). Resistant strains of poultry origin were thus collected for a further study. Several resistance profiles for the collected strains, half of which were very resistant to flumequine and to the new fluo-

roquinolones, were observed. The present study was undertaken to investigate the mechanism involved and to determine if in vitro selective pressure would give rise to resistance patterns similar to those encountered among this sample of strains.

### MATERIALS AND METHODS

**Chemicals.** Silicone oils DC 550 and DC 556 were from Touzart et Matignon, Courtaouef, France. Oil with a density of 1.043 was made by mixing 3 parts of DC 550 and 7 parts of DC 556. Topoisomerase I was purchased from Gibco BRL, Eragny, France. Plasmid pBR322 was obtained from Boehringer, Meylan, France.

**Antibiotics.** The following antibiotics were kindly provided by the indicated manufacturers: ciprofloxacin, Bayer Pharma, Sens, France; flumequine, Rikker, Malakoff, France; nalidixic acid, Winthrop, Clichy, France; norfloxacin, Merck Sharp and Dohme Chibret, Paris, France; ofloxacin, Diamant, Osny, France; and pefloxacin, sparfloxacin, and [<sup>14</sup>C]sparfloxacin, Rhône-Poulenc Rorer, Vitry-sur-Seine, France.

**Strains and isolation site.** The *E. coli* K-12 strain J53, F<sup>-</sup> pro-22 met-36, from the Institut Pasteur collection was used as a susceptible reference strain. *E. coli* DH5α from Clontech (4) was used as a nalidixic acid-resistant reference strain. It harbors the mutation Asp-87 to Asn in the A subunit of DNA gyrase (our laboratory's unpublished result), conferring resistance to nalidixic acid (MIC = 32 µg/ml).

The *E. coli* strains used in this study were isolated in Saudi Arabia from poultry clinical samples sent to a diagnostic laboratory run by one of us (M.O.). Each represented an independent isolate coming from a different flock. They belonged to the common poultry-pathogenic serovars O78 and O2 or were nontypeable. The patterns were shown by outer membrane protein analysis to all be different (data not shown) (14), confirming that the strains were independent isolates. Although it is difficult at the level of a diagnostic laboratory to obtain detailed information about antibiotic usage on the farms sending material for bacteriological analysis, the therapeutic use of oxolinic acid or flumequine in the flocks from which the clinical samples were obtained was recorded. Such treatments often had a preventive purpose and were applied to a whole flock at the onset of the first symptoms in a few animals to avoid the spread of infection or at periods when it was established that the animals were particularly susceptible to disease (e.g., risk of colibacillosis following viral infections of the respiratory tract [13]). The dosage reported for both drugs was 0.1 g/liter in the drinking water for treatment periods of 4 to 5 days, possibly repeated according to the clinical observations.

**Drug susceptibility.** MICs were measured by the agar dilution method using

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TABLE 1. Phenotypes of wild-type strains used in this study<sup>a</sup>

Class or subclass	<i>E. coli</i> strain	MIC ( $\mu\text{g/ml}$ )							
		NAL	PIPE	FLU	OFLX	NFLX	CPFAX	PFLX	SPFX
1	J53	4	2	0.5	0.06	0.25	0.03	0.25	0.06
	226	4	4	1	0.12	0.5	0.06	0.25	0.06
	193	4	2	1	0.12	0.12	0.015	0.12	0.06
2a	DH5 $\alpha$	32					0.01		
	250	32	16	4	0.25	0.5	0.12	0.5	0.12
	231	64	8	8	0.5	0.5	0.12	1	
2b	222	128	16	16	1	1	0.5	1	0.5
	307	256	16	32	1	1	0.5	2	
	251	256	16	32	1	1	0.5	2	
	266	128	16	32	1	1	0.5	1	
	182	512	16	32	1	2	1	2	1
3a	116	>2,056	>64	128	2	4	2	4	2
	181	>2,056	>64	128	2	4	2	4	
	239	>2,056	>64	128	1	4	1	4	4
3b	293	>2,056	>64	128	8	16	8	32	16
	224	>2,056	>64	128	8	16	8	32	16
3c	215	>2,056	>64	128	32	128	16	128	16
	184	>2,056	>64	128	32	128	16	128	16

<sup>a</sup> Abbreviations for drugs: NAL, nalidixic acid; PIPE, pipemidic acid; FLU, flumequine; OFLX, ofloxacin; NFLX, norfloxacin; CPFAX, ciprofloxacin; PFLX, pefloxacin; SPFX, sparfloxacin.

Mueller-Hinton agar (Pasteur Diagnostic, Paris, France) and  $10^4$  CFU per spot. Cultures were incubated for 18 h at 37°C.

**Selection of mutants.** Clones of spontaneous quinolone-resistant mutants were selected by plating overnight cultures of *E. coli* on Mueller-Hinton agar (Pasteur Diagnostic) containing from half to four times the MIC of ciprofloxacin (15). Selection was made with strains 116, 222, 226, 239, and 250.

**Accumulation assays.** Quinolone accumulation by cells was measured by centrifugation through silicone oil and a fluorescence assay, except the assay for sparfloxacin, for which  $^{14}\text{C}$ -labelled compound was used as described earlier (2). The fluorescence spectra of fluoroquinolones were recorded on an F-2000 Hitachi spectrometer; standard dosage curves were established at the maximum wavelength of each quinolone to allow further determinations of concentrations.

**Gyrase purification.** DNA gyrase was purified by affinity chromatography on novobiocin, according to the technique of Staudenbauer and Orr (28) with some modifications as described earlier (2). DNA supercoiling activity was assayed as described previously (2), with 150 ng of relaxed pBR322 plasmid and 1 U of DNA gyrase. One unit of activity is the amount of gyrase that can supercoil 150 ng of relaxed pBR322 plasmid in 30 min at 37°C. Results were expressed as the minimal effective dose (MED) (the minimum amount of drug required to cause any inhibition of activity). This parameter was easier to evaluate on electrophoresis gels than was the classical 50% effective dose and gave more accurate values.

**Transformation and complementation tests.** Plasmid pAFF801 (4), containing the *aphA1* gene and the wild-type *E. coli gyrA* gene, was used for transformation after electroporation. Transformants were selected on brain heart infusion agar containing kanamycin at 50  $\mu\text{g/ml}$  to ensure the presence of the complementing plasmid.

## RESULTS

**Drug susceptibility of bacterial isolates.** Table 1 presents the MICs of nalidixic acid, pipemidic acid, flumequine, pefloxacin, ciprofloxacin, ofloxacin, and norfloxacin for the strains used in this study. *E. coli* J53 was used as a reference susceptible strain. The isolates can be classified into three classes according to resistance to flumequine: class 1 (MIC < 4  $\mu\text{g/ml}$ ), class 2 (4  $\mu\text{g/ml}$   $\leq$  MIC < 64  $\mu\text{g/ml}$ ), and class 3 (MIC = 128  $\mu\text{g/ml}$ ). A similar system of classification was obtained for nalidixic acid: class 1 (MIC  $\leq$  4  $\mu\text{g/ml}$ ), class 2 (4  $\mu\text{g/ml}$  < MIC < 1,024  $\mu\text{g/ml}$ ), and class 3 (MIC > 1,024  $\mu\text{g/ml}$ ). The MIC profile of fluoroquinolones led us to separate the strains into six subclasses, as indicated in Table 1. In addition, bacteria were

generally resistant to tetracycline (MIC > 16  $\mu\text{g/ml}$ ), tobramycin (1  $\mu\text{g/ml}$  < MIC < 128  $\mu\text{g/ml}$ ), and trimethoprim (for 11 strains, MIC > 64  $\mu\text{g/ml}$ ) and susceptible to cefoxitin and cefotaxime. Ten of them were resistant to chloramphenicol (MICs > 256  $\mu\text{g/ml}$ ).

**Uptake of fluoroquinolones by bacterial cells.** The accumulation of 4 fluoroquinolones was measured in one strain of each subclass (strain 116, 215, 222, 226, 250, or 293). The levels of accumulation at 10 min when an external drug concentration of 10  $\mu\text{g/ml}$  was used were  $130 \pm 15$ ,  $70 \pm 10$ ,  $135 \pm 15$ , and  $50 \pm 10$  ng per  $10^9$  bacteria for ciprofloxacin, ofloxacin, pefloxacin, and sparfloxacin, respectively (values are means  $\pm$  standard deviations of three or four experiments for each strain and each quinolone assayed [results not shown]). For each quinolone, we found no more than 15% difference in accumulation between the strains, a difference that can be considered not significant (19). Under the same conditions, the reference quinolone-susceptible strain *E. coli* J53 accumulated the same amount of quinolone and a clinical strain with a low-level quinolone resistance, having lost only OmpF (10), that was taken as a negative control accumulated 30 ng of ofloxacin and 25 ng of sparfloxacin per  $10^9$  bacteria. It was therefore concluded that the strains used in this study do not demonstrate decreased accumulation of quinolones.

**Quinolone susceptibilities of transformants.** As shown in Table 2, strains 116, 215, 222, 226, 250, 293, and DH5 $\alpha$  were successfully transformed with plasmid pAFF801, carrying the wild-type *gyrA* gene. It restored the full susceptibility to fluoroquinolones to all the six transformed strains as well as to the nalidixic acid-resistant *E. coli* DH5 $\alpha$  harboring the *gyrA* mutation Asp-87 to Asn.

**Isolation of DNA gyrase.** DNA gyrase was isolated from *E. coli* J53 and from six *E. coli* strains representative of the different classes and subclasses (strains 116, 193, 215, 222, 250, and 293). The specific activities of the seven gyrases varied from 1 to  $1.5 \times 10^4$  U/mg. The MEDs of ciprofloxacin and

TABLE 2. Susceptibilities of wild-type and transformed strains used in this study<sup>a</sup>

<i>E. coli</i> strain	MIC (μg/ml)		
	NAL	CPFX	PFLX
J53	4	0.03	0.25
226	4	0.06	0.25
226(pAFF801)	2	0.02	0.1
DH5α	32	0.015	
DH5α(pAFF801)	2	0.01	0.125
250	32	0.12	0.5
250(pAFF801)	4	0.03	
222	128	0.5	1
222(pAFF801)	4	0.03	
116	>2,056	2	4
116(pAFF801)	2	0.015	0.1
293	>2,056	8	32
293(pAFF801)	4	0.06	0.1
215	>2,056	16	128
215(pAFF801)	4	0.06	0.1

<sup>a</sup> Abbreviations for drugs: NAL, naladixic acid; CPFX, ciprofloxacin; PFLX, pefloxacin.

sparfloxacin for these enzymes were determined (Table 3) and showed good correlations with the respective MICs. The gyrase from strain 215 was the most resistant to all fluoroquinolones (MED of ciprofloxacin, >90 μg/ml; MED of sparfloxacin, >350 μg/ml). All the gyrases were sensitive to novobiocin.

**Selection of fluoroquinolone-resistant mutants.** The MICs for the mutants obtained after one round of selection on ciprofloxacin using strains belonging to different classes or subclasses are shown in Table 4. Thus, 226R1 obtained from 226 showed the same level of resistance to quinolones as did 250 and 250R1 selected from 250, 222R1 selected from 222, 239R1 selected from 293, and 116R1 selected from 116 showed resistance levels similar to those of 222, 182, 224 or 293, and 215 or 184, respectively. After a second round of selection on pefloxacin, 222R2 derived from 222R1 showed a level of resistance similar to that of 239.

## DISCUSSION

The introduction of flumequine and oxolinic acid in veterinary medicine in Saudi Arabia has been followed by an apparent increase of antibiotic resistance in *E. coli* poultry clinical isolates. In the year following this introduction, the proportion of quinolone-resistant strains isolated by diagnostic laboratories increased to >50% (20a). The aim of the present study was to investigate the resistance mechanisms. We also sought to

TABLE 3. Susceptibilities of bacteria and inhibition of DNA gyrase

<i>E. coli</i> strain <sup>a</sup>	Ciprofloxacin		Sparfloxacin	
	MIC (μg/ml)	MED <sup>b</sup> (μg/ml)	MIC (μg/ml)	MED (μg/ml)
J53	0.03	0.085	0.06	0.17
193	0.015	0.17	0.06	0.17
250	0.12	0.17	0.12	0.7
222	0.5	1.4	0.5	1.4
116	2	24	2	48
293	8	96	16	190
215	16	96	16	380

<sup>a</sup> One strain representative of each subclass.

<sup>b</sup> See text for details.

TABLE 4. Susceptibilities of wild-type strains and spontaneous mutants<sup>a</sup>

<i>E. coli</i> strain	MIC (μg/ml)				
	PIPE	OFLX	CPFX	PFLX	SPFX
193	2	0.12	0.015	0.12	0.06
226	4	0.12	0.06	0.25	0.06
226R1 <sup>b</sup>	16	0.25	0.12	0.5	0.25
250	16	0.25	0.12	0.5	0.12
250R1	16	1	0.5	1	0.5
222	16	1	0.5	1	0.5
222R1	16	1	1	2	1
182	16	1	1	2	1
222R2 <sup>c</sup>	>64	2	1	4	2
239	>64	1	1	4	4
116	>64	2	2	4	2
239R1	>64	8	4	32	
293	>64	8	8	32	16
116R1	>64	32	8	128	16
215	>64	32	16	128	16

<sup>a</sup> Abbreviations for drugs: PIPE, pipemidic acid; OFLX, ofloxacin; CPFX, ciprofloxacin; PFLX, pefloxacin; SPFX, sparfloxacin.

<sup>b</sup> Spontaneous mutant selected from 226 after one round of selection.

<sup>c</sup> Spontaneous mutant selected from 222R1.

estimate the number of one-step mutations that could explain the emergence of these mutants which lead from the susceptible to the more resistant isolates.

The measure of the accumulation of five quinolones for six strains representative of each subclass indicated that the strains used in this study did not demonstrate decreased accumulation of quinolones (17). This finding suggests the possibility of a decrease in the susceptibility of DNA gyrase (16). This is in agreement with the findings of recent studies of isolates of enteric bacteria, which demonstrated that quinolone resistance among resistant *E. coli* (9) or *Salmonella typhimurium* (12, 21) isolated from clinical or veterinary specimens was not connected with changes in outer membrane proteins. Others (1, 6, 7, 14) suggested that therapeutic agents other than quinolones, such as cephalosporins, might change the bacterial outer membrane, thus producing clinical isolates with low levels of resistance to multiple antibiotics.

Following transformation with wild-type *gyrA*, the strains recovered full susceptibility to quinolones. This indicated that modifications of the subunit A of DNA gyrase occurred in these strains and was responsible for the resistance observed. The wide range of resistance levels suggested the occurrence of different and perhaps multiple mutations. The gyrases extracted from the six veterinary isolates representative of the six subclasses were all different in their levels of resistance to ciprofloxacin and sparfloxacin, and there was good agreement between the MICs and MEDs. This confirmed that modifications of gyrase were responsible for the quinolone resistance and that different mutations occurred, since we isolated gyrases with different sensitivities to quinolones. This hypothesis was confirmed by the selection of spontaneous quinolone-resistant mutants on ciprofloxacin. By using wild-type strains representative of each subclass, it was possible to select in one step a mutant with a level of resistance to quinolones similar to that of the isolates of the next-higher subclass. This indicated that some isolates may have developed more than one mutation. Similar suggestions were made by Griggs et al. (12), working on veterinary *Salmonella* spp. It has been demonstrated by others (8) that a single mutation in the gyrase A protein was solely responsible for high-level resistance to nalidixic acid (MIC = 500 μg/ml) but that the level of fluoroquinolone

resistance remained moderate (MIC = 2 µg/ml). Thus, the high level of resistance in some isolates and the inability to obtain the highly resistant mutants in one step tend to confirm this assumption. Such multiple mutations have already been described (18, 24, 33) or suggested (25) earlier. Furthermore, the possibility of double (or more) *gyrA* mutations is probable since we obtained, starting with an already-resistant strain (222), two additional levels of resistance (222R1 and 222R2). Our results indicate that, as in human medicine, cross-resistance between older quinolones and fluoroquinolones could exist in veterinary medicine and that the mechanisms responsible for the resistance are similar in both situations. Although our results strongly indicate that modifications in the A subunit of DNA gyrase may be responsible for the observed resistance patterns, one cannot exclude the possibility that additional mutations occurred. For example ParC mutations can be found in cells that are resistant to high levels of fluoroquinolones, provided GyrA is already resistant to inhibition by the antibiotic (3). The detection of fluoroquinolone resistance in a veterinary context emphasizes the need for the prudent use of such compounds.

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