

The selection of resistance to and the mutagenicity of different fluoroquinolones in *Staphylococcus aureus* and *Streptococcus pneumoniae*

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ABSTRACT

Two quinolone-susceptible *Staphylococcus aureus* and five quinolone-susceptible *Streptococcus pneumoniae* isolates were used to obtain in-vitro quinolone-resistant mutants in a multistep resistance selection process. The fluoroquinolones used were ciprofloxacin, moxifloxacin, levofloxacin, gemifloxacin, trovafloxacin and clinafloxacin. The mutagenicity of these quinolones was determined by the *Salmonella* and the *Escherichia coli* retromutation assays. All quinolone-resistant *Staph. aureus* mutants had at least one mutation in the *grrA* gene, while 86.6% of quinolone-resistant *Strep. pneumoniae* mutants had mutations in either or both the *gyrA* and *parC* genes. Moxifloxacin and levofloxacin selected resistant mutants later than the other quinolones, but this difference was more obvious in *Staph. aureus*. Accumulation of the fluoroquinolones by *Staph. aureus* did not explain these differences, since levofloxacin and moxifloxacin accumulated inside bacteria to the same extent as clinafloxacin and trovafloxacin. The results also showed that moxifloxacin and levofloxacin had less mutagenic potency in both mutagenicity assays, suggesting a possible relationship between the selection of resistance to quinolones and the mutagenic potency of the molecule. Furthermore, gemifloxacin selected efflux mutants more frequently than the other quinolones used. Thus, the risk of developing quinolone resistance may depend on the inoculum of the microorganism at the infection site and the concentration of the fluoroquinolone, and also on the mutagenicity of the quinolone used, with moxifloxacin and levofloxacin being the least mutagenic.

Keywords Fluoroquinolones, mutagenicity, quinolone, resistance selection, *Staphylococcus aureus*, *Streptococcus pneumoniae*

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INTRODUCTION

The use of quinolones has risen steadily since their introduction. The newer fluoroquinolones, such as levofloxacin and moxifloxacin, have enhanced activity against Gram-positive microorganisms, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, and have therefore been used mainly to treat respiratory tract infections [1]. Mechanisms of resistance to quinolones in

Gram-positive bacteria have been classified into two groups: (i) mutations in the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes (*grrA* in *Staph. aureus*), which encode the A subunits of DNA gyrase and topoisomerase IV, respectively [2–8]; and (ii) efflux systems that pump the drug out of the cell [9–12]. Efflux systems can be overexpressed following mutations in the gene encoding the protein that regulates the expression of the efflux system, mutations in the gene encoding the efflux protein itself, resulting in a greater ability to extrude quinolones [13], or a mutation in the promoter region [14]. Overall, resistance can be associated with either or both of these

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mechanisms. In-vivo selection of quinolone resistance in bacteria is influenced by: (i) the bacterial species involved; (ii) the quinolone used; (iii) the concentration of antibiotic at the site of infection, and the ratio between this concentration and the MIC of the antibiotic for the microorganism; and (iv) the density of bacteria at the site of infection. Several studies have shown that different fluoroquinolones have different potentials to select resistance [15–18].

The mutagenic effect of nalidixic and oxolinic acids on bacteria was first demonstrated in the *Salmonella* mutagenicity assay [19]. Using this assay, this observation was further expanded to enoxacin and ciprofloxacin [20], fleroxacin and enrofloxacin [21], norfloxacin, temafloxacin, tosufloxacin and lomefloxacin [22], and other DNA gyrase and mammalian topoisomerase II inhibitors [23]. In these studies, reversion of the *hisG428* ochre mutation in strain TA102 was the genetic endpoint used, since other *Salmonella* tester strains failed to detect the mutagenic effect of these antibacterial compounds [20]. The mutagenic effect of nalidixic and oxolinic acids has also been detected in the *Escherichia coli* WP2 *trp*⁺ reversion assay [24,25]. It has been suggested that mutagenicity of quinolones could be one of the reasons for the emergence of resistant clinical strains [22,26]. Moreover, it has been shown that adaptive mutations produce resistance to ciprofloxacin in *E. coli* [27]. Therefore, the present study aimed to investigate the mutagenic potency and potential of six quinolones to select for resistance in *Staph. aureus* and *Strep. pneumoniae*.

MATERIALS AND METHODS

Microorganisms and susceptibility testing

The study investigated two *Staph. aureus* and five *Strep. pneumoniae* clinical isolates. The MICs for the clinical isolates and the derivative mutants were determined by the NCCLS microdilution method [28] in the absence and presence of reserpine (25 mg/L). *Staph. aureus* was grown in Mueller–Hinton cation-adjusted broth, which was supplemented with lysed horse blood 5% v/v for the growth of *Strep. pneumoniae*.

Selection of mutants by serial passages

Resistance was selected by serial passages performed with antibiotic concentrations ranging from three doubling dilutions below to three doubling dilutions above the MIC. Based on the method described by Browne *et al.* [29], with slight modifications, strains were subcultured on blood agar without antibiotic pressure, instead of cation-adjusted Mueller–Hinton

broth supplemented with horse blood, before the next antibiotic passage. Daily subculturing was performed until mutants with MICs that were $\geq 4\times$ the MIC of the selected drug were obtained. The antimicrobial agents used in this study were ciprofloxacin and moxifloxacin (Bayer, Leverkusen, Germany), levofloxacin (Aventis, Madrid, Spain), gemifloxacin (Glaxo-SmithKline, Harlow, UK), trovafloxacin (Pfizer Ltd, Sandwich, UK) and clinafloxacin (Parke-Davis, Ann Arbor, MI, USA). The ability of the fluoroquinolones to select resistant mutants was defined as the number of passages necessary to increase the initial MIC four-fold.

Mutations in the target genes

For each of the clinical isolates investigated, the QRDRs of the *gyrA*, *gyrB*, *parC* and *parE* genes (*griA* and *griB* for *Staph. aureus*) were amplified by PCR [5,8] and sequenced to ensure that none of the isolates had a mutation associated with decreased susceptibility. PCR products were purified with a QiaQuick PCR purification kit (Qiagen, Hilden, Germany) and sequenced with a Big Dye terminator sequence kit v. 2.0 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Mutations in the QRDRs of the *gyrA* and *parC* genes of the mutants selected were analysed by PCR–restriction fragment length polymorphism with *Hin*I (Ser84 of GyrA and Ser80 of GriA of *Staph. aureus*; Ser81 of GyrA and Ser79 of ParC of *Strep. pneumoniae*), except for some resistant mutants of *Staph. aureus* with a ciprofloxacin MIC of 32 or 64 mg/L, for which the QRDRs of the *gyrA* and *griA* genes were sequenced.

Accumulation assay

The λ_{ex} and λ_{em} of each quinolone were determined for the clinical isolates of *Staph. aureus* with a modified fluorimetric method, as described by Mortimer and Piddock [30] and Martinez-Martinez *et al.* [31]. Briefly, cells were grown to the exponential phase (OD₆₀₀ of 0.6–0.7), pelleted by centrifugation, washed with phosphate-buffered saline, and then resuspended in phosphate-buffered saline to an OD₅₂₀ of 1.5. Aliquots (10 μ L) of a quinolone dilution were added to 490 μ L of bacterial suspension and incubated for 30 min, after which the bacterial cells were again pelleted and washed twice with phosphate-buffered saline. The final pellet was resuspended in glycine-HCl, pH 3.0, and incubated for 2 h at room temperature. Finally, the cells were pelleted, the supernatant was transferred to a new tube, and the amount of fluorescence was measured. Calibration curves were also constructed for each quinolone.

Mutagenesis assays

Two reversion mutation methods were used. The *Salmonella* mutagenicity assay was performed by the plate incorporation method with the mutant strain TA102, as described previously, with three plates per concentration [32]. The *E. coli* WP2 *trp*⁺ reversion assay [33] was performed with the mutant strain WP2/pKM101 [24] according to recent recommendations [34]. The number of revertants induced was calculated by subtracting the number of spontaneous revertants obtained in the negative control for each experiment. Linear regression was calculated from the data of three independent experiments with non-toxic doses, with the mutagenic potency being the slope of the line obtained for each compound.

RESULTS

Multistep selection of resistance

The ability of several fluoroquinolones to select resistant mutants of *Staph. aureus* and *Strep. pneumoniae* by serial passages is shown in Tables 1 and 2. Ciprofloxacin was the first antimicrobial agent to

select resistance (5–8 passages for *Staph. aureus*; 7–10 passages for *Strep. pneumoniae*), followed, for *Staph. aureus*, by trovafloxacin (6–8 passages) and gemifloxacin (8 passages). For *Strep. pneumoniae*, trovafloxacin and gemifloxacin selected resistance following 7–11 and 8–10 passages, respectively. Clinafloxacin selected resistance following 8–11

Table 1. MICs (mg/L) of quinolones in the presence and absence of reserpine for the selected mutants of *Staphylococcus aureus*

Wild-type strain	Mutants	<i>gyrA</i> Ser84	<i>griA</i> Ser80	Original MIC ^a	C	C + R ^b	M	M + R ^b	L	L + R ^b	G	G + R ^b	T	T + R ^b	Clx	Clx + R ^b
4-108	4-108C5	Wild-type	Mutant	0.06	2	1	≤ 0.125	< 0.03	0.5	0.25	≤ 0.06	0.015	≤ 0.125	0.06	≥ 0.125	0.03
	4-108M16	Wild-type ^c	Mutant	0.03	64	64	1	0.5	8	8	1	0.5	1	1	1	0.25
	4-108L13	Wild-type ^c	Mutant	0.125	64	32	0.5	0.5	8	4	1	0.5	1	0.25	0.5	0.25
	4-108G8	Wild-type	Mutant	0.007	4	4	≤ 0.125	0.25	0.5	0.5	≤ 0.06	0.125	≤ 0.125	0.25	≥ 0.125	0.125
	4-108T6	Wild-type	Mutant	0.015	32	2	0.5	0.125	4	0.5	0.5	0.03	0.25	0.125	0.5	0.06
5-61	4-108Clx8	Wild-type	Mutant	0.015	4	2	0.25	0.25	1	0.5	≤ 0.06	0.125	0.25	0.125	0.25	0.125
	5-61C7	Wild-type	Mutant	0.06	2	1	≤ 0.03	< 0.03	0.25	0.25	≤ 0.007	≤ 0.007	0.03	0.03	0.007	< 0.015
	5-61M14	Wild-type ^c	Mutant	0.03	64	64	0.5	0.5	8	4	1	0.5	0.5	0.5	0.5	0.25
	5-61L15	Wild-type ^c	Mutant	0.125	32	32	0.5	0.5	8	4	1	0.5	0.5	0.5	0.5	0.25
	5-61G8	Wild-type	Mutant	0.007	8	1	0.125	0.125	0.5	0.5	0.06	0.03	0.125	0.125	0.06	0.06
	5-61T8	Wild-type	Mutant	0.015	2	2	0.25	0.25	0.5	1	0.06	0.06	0.25	0.25	0.06	0.06
	5-61Clx11	Wild-type	Mutant	0.015	4	4	0.25	0.25	2	1	0.125	0.06	0.25	0.25	0.125	0.06

Example of strain nomenclature: 4-108, number of strain; C, antibiotic used for selection; 5, number of passages required for selection.

C, ciprofloxacin; M, moxifloxacin; L, levofloxacin; G, gemifloxacin; T, trovafloxacin; Clx, clinafloxacin; R, reserpine.

^aThe MIC of the selecting antibiotic for the wild-type clinical isolate.

^bMICs obtained in the presence of reserpine 25 mg/L.

^cThese strains had an additional mutation at codon Glu-88, changing it to Lys.

Table 2. MICs (mg/L) of quinolones in the presence and absence of reserpine for the selected mutants of *Streptococcus pneumoniae*

Wild-type strain	Mutant	<i>gyrA</i> Ser81	<i>parC</i> Ser79	Original MIC ^a	C	C + R ^b	M	M + R ^b	L	L + R ^b	G	G + R ^b	T	T + R ^b	Clx	Clx + R ^b
5-1	5-1C10	Mutant	Mutant	1	32	32	4	2	16	16	0.25	0.25	2	2	1	0.5
	5-1M12	Mutant	Mutant	0.125	32	16	2	2	16	16	0.25	0.25	2	2	0.5	0.5
	5-1L12	Mutant	Mutant	1	64	32	4	2	16	8	0.5	0.25	4	4	1	0.5
	5-1G9	Wild-type	Mutant	0.015	16	1	1	0.125	4	1	0.25	< 0.015	0.5	0.125	0.5	< 0.06
	5-1T12	Mutant	Mutant	0.06	16	16	8	4	16	16	0.5	0.25	2	4	1	0.5
5-4	5-1Clx14	Mutant	Wild-type	0.06	32	8	8	1	16	16	0.5	0.25	2	1	2	0.25
	5-4C7	Mutant	Wild-type	0.5	32	8	4	0.5	8	4	1	0.25	1	0.5	1	0.25
	5-4M9	Mutant	Mutant	0.25	32	16	8	4	32	16	1	0.25	4	4	1	0.5
	5-4L9	Mutant	Wild-type	1	16	8	4	0.5	32	16	0.5	0.125	2	1	2	0.5
	5-4G8	Wild-type	Wild-type	0.015	8	1	0.25	0.25	4	1	0.125	0.03	0.25	0.125	0.25	< 0.06
	5-4T7	Wild-type	Mutant	0.06	4	2	0.5	0.25	2	2	0.125	0.06	0.5	0.25	0.125	< 0.06
	5-4Clx8	Wild-type	Wild-type	0.06	16	0.5	0.5	0.125	2	1	0.125	< 0.015	0.25	0.125	0.5	< 0.06
5-6	5-6C7	Mutant	Wild-type	1	32	8	4	1	16	8	1	0.125	2	0.5	1	0.125
	5-6M9	Mutant	Mutant	0.125	16	16	4	4	16	16	0.5	0.25	4	4	0.5	0.25
	5-6L12	Mutant	Wild-type	1	32	8	8	2	16	16	1	0.125	2	2	2	0.5
	5-6G8	Wild-type	Wild-type	0.015	16	1	1	0.5	4	2	0.25	0.06	0.25	0.125	0.5	< 0.06
	5-6T11	Mutant	Mutant	0.06	64	8	8	4	64	8	2	0.5	4	2	4	1
5-11	5-6Clx9	Wild-type	Wild-type	0.06	4	0.5	0.5	0.125	2	1	0.06	< 0.015	0.25	0.125	0.125	< 0.06
	5-11C7	Mutant	Wild-type	1	32	4	4	2	16	2	1	0.125	1	0.25	1	0.25
	5-11M9	Mutant	Mutant	0.125	64	8	4	2	32	2	0.5	0.25	4	2	1	0.25
	5-11L13	Mutant	Wild-type	1	16	4	2	1	16	4	0.5	0.125	2	1	2	0.5
	5-11G10	Wild-type	Mutant	0.015	32	1	0.5	0.25	4	0.25	0.25	< 0.015	0.5	0.25	0.25	< 0.06
	5-11T11	Mutant	Mutant	0.06	8	8	4	4	8	8	0.25	0.125	2	1	0.5	0.125
	5-11Clx12	Wild-type	Mutant	0.06	64	2	8	4	32	4	4	0.5	16	4	8	1
5-154	5-154C7	Mutant	Wild-type	0.5	64	4	2	1	8	1	1	0.25	1	0.5	1	0.25
	5-154M8	Mutant	Mutant	0.06	32	8	4	4	16	4	0.25	0.125	2	2	0.5	0.25
	5-154L13	Mutant	Wild-type	1	32	4	4	2	16	2	0.5	0.125	2	2	1	1
	5-154G10	Wild-type	Mutant	0.015	32	0.5	0.5	0.125	4	0.125	0.25	< 0.015	0.5	0.125	0.5	< 0.06
	5-154T11	Mutant	Mutant	0.06	64	8	8	4	32	8	4	0.5	8	2	8	1
5-154Clx12	Mutant	Mutant	0.06	64	8	4	4	32	4	2	0.25	4	4	2	0.25	

Example of strain nomenclature: 5-1, number of strain; C, antibiotic used for selection; 10, number of passages required for selection.

C, ciprofloxacin; M, moxifloxacin; L, levofloxacin; G, gemifloxacin; T, trovafloxacin; Clx, clinafloxacin; R, reserpine.

^aThe MIC of the selecting antibiotic for the wild-type clinical isolate.

^bMICs obtained in the presence of reserpine 25 mg/L.

Previous mutations in wild-type strains: strain 5-1, no mutation; strain 5-4, mutation in ParC, R95-C; strain 5-6, mutation in ParC, K137-N; strain 5-11, mutation in ParE, I368-V; strain 5-154, mutation in GyrA, E85-K.

passages in *Staph. aureus*, and after 8–12 passages in *Strep. pneumoniae*. The fluoroquinolones that needed most passages to select resistant mutants in *Staph. aureus* were moxifloxacin and levofloxacin (14–16 and 12–15 passages, respectively). In *Strep. pneumoniae*, moxifloxacin selected resistant mutants after 8–12 passages, whereas levofloxacin required 9–13 passages.

Mutations in target genes

No mutations were found in the QRDRs of the *gyrA*, *gyrB*, *griA* or *griB* genes in the wild-type strains of *Staph. aureus*, whereas several different mutations were found in the wild-type strains of *Strep. pneumoniae*. Strain 5-1 did not have any mutation, strain 5-4 contained an alteration in ParC (R95-C), strain 5-6 had a different substitution in ParC (K137-N), strain 5-11 had an amino-acid change at ParE (I368-V), and strain 5-154 had a mutation in the amino-acid codon E85-K of the *gyrA* gene. All of these strains were susceptible to ciprofloxacin.

All *Staph. aureus* mutants had a mutation in the Ser80 codon of the *griA* gene, but no mutation was detected in the Ser80 codon of the *gyrA* gene. The *gyrA* and *griA* genes of four mutants (4-108L13, 4-108M16, 5-61L15 and 5-61M14) with a ciprofloxacin MIC of 32 or 64 mg/L were sequenced, revealing a mutation in the Glu88 codon of the *gyrA* gene, producing a change from Glu to Lys. In *Strep. pneumoniae*, 26 of 30 resistant mutants had mutations in either *gyrA* or *parC*, or both. Twelve (40%) of the *Strep. pneumoniae* mutants had a double mutation, with a mutation in the Ser81 codon of the *gyrA* gene, plus a mutation in the Ser79 codon of the *parC* gene (genotype 1); nine (30%) had a mutation in the *gyrA* gene, but not in the *parC* gene (genotype 2); five (16.6%) had a mutation in the *parC* gene, but not in the *gyrA* gene (genotype 3); and four (13.3%) did not have mutations in either gene at these two codons (genotype 4). Two of the latter four strains were selected with clinafloxacin, and the remaining two with gemifloxacin (Table 1). The distribution of the mutations selected by each antibiotic differed; thus, ciprofloxacin and levofloxacin selected four mutants with genotype 2 and one with genotype 1; moxifloxacin only selected genotype 1; gemifloxacin selected two mutants with genotype 4, and three with genotype 3; trovafloxacin selected one mutant with genotype

3 and four with genotype 1; and clinafloxacin selected two mutants with genotype 4, and one each of the other genotypes.

Susceptibility testing

All of the resistant mutants obtained had an MIC at least four-fold higher than the MIC of the fluoroquinolone used for their selection, while the MICs of the other fluoroquinolones increased by different degrees. MICs in the presence of reserpine were also determined, to find whether an efflux system inhibited by reserpine was involved in the resistance exhibited by the mutants (Tables 1 and 2).

In general, the MICs for *Staph. aureus* did not change in the presence of reserpine, but four mutants showed a decreased MIC: strain 4-108C5 showed a four-fold decrease in the MIC of moxifloxacin; strain 4-108M16 showed a four-fold decrease in the MIC of clinafloxacin; strain 5-61G8 showed an eight-fold decrease in the MIC of ciprofloxacin; and strain 4-108T6 showed at least a four-fold decrease in the MIC to each quinolone used, with the exception of trovafloxacin (Tables 1 and 2).

For *Strep. pneumoniae*, different efflux profiles were observed in the presence of reserpine, depending on the genotype of the mutant obtained (Table 3). The first group of four strains with no detectable mutations (genotype 4) comprised two strains obtained with gemifloxacin and two with clinafloxacin. Three of these four had the same efflux-pump profile, affecting the same quinolones. In all four strains, the MICs in the presence of reserpine decreased to the MICs of the respective wild-type strains, or to a maximum of twice the wild-type MIC for some quinolones. In the second group of five strains (genotype 3), there were four different profiles (Table 3). In these strains, the MIC in the presence of reserpine decreased to a mean of 2–4 × the MIC of their respective wild-type strains. The third group (genotype 2) contained nine strains with seven different profiles (Table 3). In this group, the MICs of the mutants in the presence of reserpine were 8–16-fold greater than those for the respective wild-type strains. Finally, the last group (genotype 1) contained 12 strains with six different efflux profiles, with 50% of the strains showing a profile characterised by a lack of any effect of reserpine on the MICs of the different

Table 3. Effect of growth in the presence of reserpine on antibiotic MICs for *Streptococcus pneumoniae* mutants

Mutant	Genotype	C	M	L	G	T	Clx	Efflux profile
5-4G8	4	+	-	+	+	-	+	A
5-4Clx8	4	+	+	-	+	-	+	B
5-6G8	4	+	+	-	+	-	+	B
5-6Clx9	4	+	+	-	+	-	+	B
5-1G9	3	+	+	+	+	+	+	A
5-4T7	3	-	-	-	-	-	+/-	B
5-11G10	3	+	-	+	+	-	+	C
5-11Clx12	3	+	-	+	+	+	+	D
5-154G10	3	+	+	+	+	+	+	A
5-1Clx14	2	+	+	-	-	-	+	A
5-4C7	2	+	+	-	+	+	+	B
5-4L9	2	-	+	-	+	-	+	C
5-6C7	2	+	+	-	+	+	+	D
5-6L12	2	+	+	-	+	-	+	E
5-11C7	2	+	-	+	+	+	+	F
5-11L13	2	+	-	+	+	-	+	G
5-154C7	2	+	-	+	+	-	+	G
5-154L13	2	+	-	+	+	-	+	G
5-1C10	1	-	-	-	-	-	-	A
5-1M12	1	-	-	-	-	-	-	A
5-1L12	1	-	-	-	-	-	-	A
5-1T12	1	-	-	-	-	-	-	A
5-4M9	1	-	-	-	+	-	-	B
5-6M9	1	-	-	-	-	-	-	A
5-6T11	1	+	-	+	+	-	+	C
5-11M9	1	+	-	+	-	-	+	D
5-11T11	1	-	-	-	-	-	+/-	A
5-154M8	1	+	-	+	-	-	-	E
5-154T11	1	+	-	+	+	+	+	F
5-154Clx12	1	+	-	+	+	-	+	C

Example of strain nomenclature: 5-4, number of strain; G, antibiotic used for selection; 8, number of passages required for selection.

C, ciprofloxacin; M, moxifloxacin; L, levofloxacin; G, gemifloxacin; T, trovafloxacin; Clx, clinafloxacin.

+, MIC decreased at least four-fold in the presence of reserpine.

-, MIC not affected in the presence of reserpine.

quinolones tested. This profile was only found in this genotype group (Table 3). These strains had the highest MICs in the presence of reserpine, with MICs that were ≥ 32 -fold those of the respective wild-type strains.

Overall, the effect of reserpine on the MICs of the different fluoroquinolones was a decrease in the MIC for 76% of the strains with clinafloxacin, for 70% with ciprofloxacin, for 66% with gemifloxacin, for 46% with levofloxacin, for 30% with moxifloxacin, and for 23% with trovafloxacin.

Fluoroquinolone accumulation in *Staph. aureus*

The results of the accumulation assay are summarised in Table 4. Ciprofloxacin and gemifloxacin showed the greatest accumulation, followed by moxifloxacin, and finally levofloxacin,

Table 4. Accumulation (ng of antimicrobial agent/mg dry weight of bacteria) of six fluoroquinolones by two wild-type strains of *Staphylococcus aureus*

Strain	C	M	L	G	T	Clx
4-108	237.96 \pm 7.57	71.45 \pm 5.91	32.91 \pm 3.75	168.82 \pm 6.46	48.04 \pm 4.65	24.25 \pm 3.62
5-61	234.47 \pm 5.48	98.32 \pm 5.74	46.13 \pm 3.88	187.64 \pm 9.51	21.06 \pm 3.97	37.50 \pm 5.55

C, ciprofloxacin; M, moxifloxacin; L, levofloxacin; G, gemifloxacin; T, trovafloxacin; Clx, clinafloxacin.

trovafloxacin and clinafloxacin, all of which showed least accumulation at the same rate.

Mutagenicity

Mutation reversion assays with *Salmonella* TA102 and *E. coli* WP2/pKM101 were performed using a wide range of concentrations (0.00312–0.1 $\mu\text{g}/\text{plate}$) of the six quinolones. A dose-response relationship was observed with both assays, while quinolone toxicity was observed at certain doses, through a decrease in the number of revertants/plate and by examination of the background lawn of bacteria on the plates. In the *E. coli* assay, toxicity was observed with 0.025 $\mu\text{g}/\text{plate}$ of moxifloxacin, and with 0.0125 $\mu\text{g}/\text{plate}$ of the other quinolones. In contrast, *Salmonella* TA102 appeared to be less sensitive to the bactericidal activity of quinolones, as the toxic effect was detected with 0.025 $\mu\text{g}/\text{plate}$ of trovafloxacin, gemifloxacin and clinafloxacin, and with 0.05 $\mu\text{g}/\text{plate}$ of the other compounds. The quinolone-induced reversion at non-toxic doses is shown in Fig. 1 for both assays, and indicates that *Salmonella* was more sensitive than *E. coli* in detecting the mutagenic effect of all the quinolones tested. In order to quantify the mutagenic potential of quinolones, the mutagenic potency, expressed as the number of induced revertants/ μg quinolone/plate (Table 5), was calculated from the data shown in Fig. 1. In the *E. coli* assay, levofloxacin and moxifloxacin were the least mutagenic compounds, while the rest of the quinolones were 1.5–2.1-fold more mutagenic than moxifloxacin. In the *Salmonella* assay, levofloxacin, moxifloxacin and ciprofloxacin were the least mutagenic compounds, while clinafloxacin, gemifloxacin and trovafloxacin were *c.* three-fold more mutagenic than levofloxacin.

DISCUSSION

In Gram-positive bacteria, mutations at the QRDRs of both DNA gyrase and topoisomerase IV are the best-known mechanisms conferring resistance to quinolones. However, efflux systems

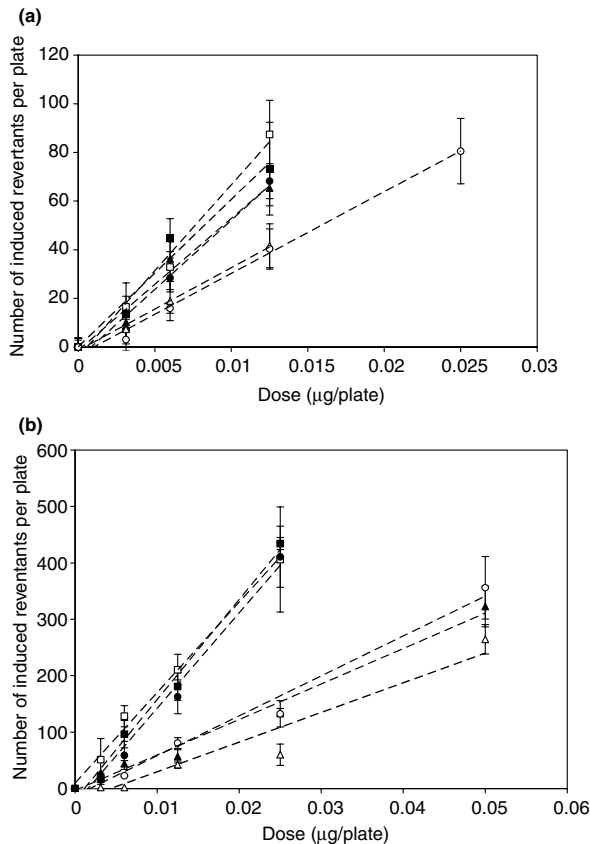


Fig. 1. Dose-mutation relationship induced by ciprofloxacin (▲), clinafloxacin (□), gemifloxacin (●), levofloxacin (△), moxifloxacin (○) and trovafloxacin (■) in the (a) *Escherichia coli* and (b) *Salmonella* assays. Standard deviations calculated from three independent experiments are shown.

Table 5. Mutagenic potency of each quinolone in *E. coli* WP2/pKM101 and *Salmonella* TA102

Quinolone	Mutagenic potency ^a	
	<i>Escherichia coli</i>	<i>Salmonella</i>
Ciprofloxacin	5423	6279
Levofloxacin	3401	5279
Moxifloxacin	3360	7071
Clinafloxacin	7070	16 034
Gemifloxacin	5689	16 884
Trovafloxacin	6060	17 742

^aNumber of induced mutants/mg/plate.

that pump quinolones out of the cells also seem to play an important role in the acquisition of resistance in *Strep. pneumoniae* and *Staph. aureus*. The mechanism by which each individual quinolone selects resistant strains is still uncertain, but may depend on the bacterial species, the quinolone used and the density of the bacteria at the

infection site. The present study is the first to correlate the acquisition of quinolone resistance with the mutagenic potency of quinolones.

Several reports have described the low frequency at which levofloxacin and moxifloxacin select for quinolone-resistant mutants of *Strep. pneumoniae* [15–17]. Nagai *et al.* [35] observed that ciprofloxacin selected resistant mutants of *Strep. pneumoniae* in a small number of steps, and that clinafloxacin and trovafloxacin behaved in a similar manner. The present study also found that more steps were required for levofloxacin and moxifloxacin to select resistant strains than were required with ciprofloxacin, gemifloxacin, trovafloxacin and clinafloxacin. Similar results were obtained with *Staph. aureus*, with all the quinolones selecting at least one mutation in the Ser80 codon of GrlA. There was a broad range of MICs for these mutants; for example, the ciprofloxacin MICs were 2–64 mg/L in the absence of reserpine, and 1–64 mg/L in the presence of reserpine, suggesting the possibility of other mutations in the target genes, or the presence of efflux systems not inhibited by reserpine in the mutants with higher MICs. A ciprofloxacin MIC of 32–64 mg/L was associated with mutations in the *gyrA* and *grrA* genes. A mutation in the Glu88 codon of GyrA, changing Glu to Lys, and a mutation in the Ser80 codon of GrlA, changing Ser to Phe, were detected.

The effect of the efflux systems inhibited by reserpine was less evident in *Staph. aureus* than in *Strep. pneumoniae*. Thus, only one mutant, obtained with trovafloxacin, showed a decreased MIC of each quinolone in the presence of reserpine. These results are in agreement with those of Boos *et al.* [15], who selected a resistant mutant after subculturing *Staph. aureus* for 10 days in sub-inhibitory concentrations of quinolones and did not observe any inhibitory effect of reserpine on efflux systems.

The fluoroquinolones used in the present study selected resistant mutants of *Strep. pneumoniae* at different frequencies, with different mutations in target genes. Strains belonging to genotype 4 had at least a four-fold increase in MIC of the selecting quinolone, which was reduced to that of the wild-type strain in the presence of reserpine, suggesting the presence of an efflux system. For genotype 3, the MICs also decreased to those of the wild-type strains, or remained at only twice the MIC of the wild-type strains, in the presence of reserpine,

suggesting that a mutation in Ser79 of ParC only generates a low level of quinolone resistance (about twice the MIC) and that the final MIC of the mutants is partially caused by the mutation in the *parC* gene and the overexpression of an efflux pump. For genotype 2, the MICs in the presence of reserpine remained at least eight-fold higher than those of the wild-type strains, suggesting that the final MIC is modulated by a combination of a mutation in the *gyrA* gene and the overexpression of a reserpine-inhibited efflux pump. The high MICs observed for this genotype suggest that a mutation in *gyrA* allows the bacteria to achieve higher fluoroquinolone resistance levels than those resulting from a mutation in *parC*.

Finally, the MICs of most of the mutants belonging to genotype 1 were not affected by reserpine, with only a two-fold decrease in MIC being observed for the strains in which reserpine did have an effect. It was interesting to note that gemifloxacin selected for the overexpression of efflux systems more frequently than did the other quinolones, as has been reported previously [15].

Accumulation of the different quinolones was affected in different ways by the efflux systems. Thus, clinafloxacin was most affected, followed by ciprofloxacin, gemifloxacin, levofloxacin, moxifloxacin, and finally trovafloxacin. Based on the different efflux profiles (Table 3), more than one efflux system may be implicated in the acquisition of resistance to quinolones. Novel efflux systems, other than NorA and PmrA, have been described in *Staph. aureus* and *Strep. pneumoniae* [36,37]. Furthermore, different efflux systems could have different affinities for each quinolone [36], and the overexpression of one or more efflux systems concomitantly may affect various quinolones differently, so that the addition of reserpine may result in the demonstration of different efflux profiles.

The results obtained in this study clearly indicate that the mutagenic potency of the quinolones studied is variable, with moxifloxacin and levofloxacin having the lowest mutagenic potency. The data suggest a correlation between the mutagenic potency of each quinolone and the ability of the molecule to select for resistant mutants. Different rates of accumulation of these compounds inside bacterial cells could explain the differences found among the different quinolones in respect of both effects. However, reported data on quinolone accumulation in different

species, and also the results obtained in the present study, do not support this hypothesis. Piddock and Johnson [38] reported that ciprofloxacin showed the greatest accumulation in *Strep. pneumoniae* cells, while levofloxacin, moxifloxacin, clinafloxacin and trovafloxacin had the same rate of accumulation. Similar results have been obtained in the present study for *Staph. aureus*, with ciprofloxacin and gemifloxacin clearly showing the highest accumulation, followed by moxifloxacin, and finally, levofloxacin, trovafloxacin and clinafloxacin. These data may explain why ciprofloxacin selects resistant mutants more rapidly than do other quinolones. However, they do not clarify why clinafloxacin and trovafloxacin select mutants more quickly than levofloxacin or moxifloxacin. One possibility is that the ability of each quinolone to select resistant mutants is related partially to the mutagenic potency of the quinolone. This potential depends on both the particular molecule and the bacteria. In both mutagenicity assays, trovafloxacin, gemifloxacin and clinafloxacin were the quinolones with the highest mutagenic potency, while moxifloxacin and levofloxacin were the least mutagenic, with the mutagenic potency of ciprofloxacin showing variation between the assays. Moxifloxacin and levofloxacin contain an alcoxyl group at position 8, while moxifloxacin and levofloxacin contain a methoxyl and propoxyl group, respectively. This agrees with the observation that a C8-methoxyl group in the quinolone molecule reduces the selection of resistant mutants in *Mycobacterium bovis* at low doses [39]. If a pathogenic strain possesses the genetic requirements for SOS mutagenesis, exposure to low concentrations of quinolones may generate mutations that confer antibiotic resistance, and these pre-existing mutations can emerge subsequently following selection as a consequence of antibiotic treatment. Thus, the risk of the development of quinolone resistance may depend on the mutagenic potency of each quinolone, with moxifloxacin and levofloxacin having the least mutagenic effect.

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