

Mutant Prevention Concentrations of Fluoroquinolones for *Enterobacteriaceae* Expressing the Plasmid-Carried Quinolone Resistance Determinant *qnrA1*[∇]

J. M. Rodríguez-Martínez,^{1*} C. Velasco,¹ I. García,¹ M. E. Cano,³
L. Martínez-Martínez,³ and A. Pascual^{1,2}

Department of Microbiology, University of Seville, Seville,¹ Service of Microbiology, University Hospital Virgen Macarena, Seville,²
and Service of Microbiology, University Hospital Marqués de Valdecilla, Santander,³ Spain

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The influence of *qnrA1* on the development of quinolone resistance in *Enterobacteriaceae* was evaluated by using the mutant prevention concentration parameter. The expression of *qnrA1* considerably increased the mutant prevention concentration compared to strains without this gene. In the presence of *qnrA1*, mutations in *gyrA* and *parC* genes were easily selected to produce high levels of quinolone resistance.

Fluoroquinolone resistance in *Enterobacteriaceae* is usually the result of mutations in chromosomal genes for type II topoisomerases or in genes coding for or regulating efflux pumps and/or porins (6). Recent reports indicate that quinolone resistance may also be plasmid mediated by the *qnrA*, *qnrS*, *qnrB*, or *aac(6′)-Ib-cr* genes (12, 16). Although *qnrA* (and, presumably, related) genes confer low-level resistance on their own, when found in the presence of other mechanisms, they are able to help select for mutants with increased levels of fluoroquinolone resistance (6, 11, 16).

A major goal of antimicrobial therapy is to achieve sufficient drug exposure in relation to MIC for optimal efficacy at the site of infection. Activity can be assessed by using the mutant prevention concentration (MPC). This is the drug concentration that prevents the growth of the least-susceptible single-step mutant present in a large bacterial population, taking into account resistant mutant subpopulations present prior to antimicrobial treatment (1, 5). The mutant selection window, the antibiotic concentration found between MIC and MPC, is where single-step mutants can be enriched (5).

The aim of the present study was to determine the influence of *qnrA1* on the development of quinolone resistance in *Enterobacteriaceae* using the MPC parameter. For this purpose, 14 strains were evaluated. These included: (i) four *Klebsiella pneumoniae* clinical strains containing *qnrA1* with different levels of fluoroquinolone susceptibility (Tables 1 and 2) (11, 17) and (ii) three groups of isogenic strains that both express and do not express *qnrA1* (Table 1). The primers used to clone *qnrA1* in pACYC184 (New England Biolabs, Inc., Barcelona, Spain) were 5′-CGGCAGTTAAAATTGGGGCT-3′ and 5′-GACCAGACTGCATAAGCAACAC-3′.

qnrA1 expression as the only quinolone resistance mechanism did not bring about fluoroquinolone resistance; however, the association of *qnrA1* with other mechanisms, such as porin

loss and Ser83Phe substitution in the GyrA protein of the *K. pneumoniae* C2 pMG252 strain, did produce a phenotype of intermediate fluoroquinolone susceptibility, which supported the additive nature of the mechanism (Table 2) (10). This could imply an important tool in the acquisition of high quinolone resistance levels in relation to plasmid-mediated quinolone resistance (14, 17, 21).

MPC assays were performed on all 14 strains, as previously described (9), permitting colony growth for up to 96 h. Most strains required between 48 and 96 h of incubation before resistant colonies were observable, except in the N5 and 1960 strains for moxifloxacin and in the C2 and C2/pMG252 strains for ciprofloxacin and moxifloxacin (Table 2). The MPC values of each fluoroquinolone for the different strains expressing the *qnrA1* gene as the only known quinolone resistance mechanism (*Escherichia coli* DH10B+*qnrA1*; *E. coli* J53 transconjugants containing *qnrA1*) reached levels (2 to 4 μg/ml) approaching the peak serum concentrations attained during therapy (Table 2) (5, 7, 20). MPC values for the non-*qnrA1*-producing isogenic controls ranged from 0.015 to 0.125 μg/ml (Table 2). These values were much higher in strains containing additional resistance mechanisms. The MPCs of clinical *qnrA1*-containing *K. pneumoniae* strains ranged from 4 to 64 μg/ml for the various fluoroquinolones. MPC values for *K. pneumoniae* C2 and *K. pneumoniae* C2 pMG252 strains were 4 to 8 μg/ml and 32 to 128 μg/ml, respectively, showing, once again, the effect of the *qnrA1* gene on the MPC parameter. A potential consequence of a suboptimal therapy selecting for resistance can be exemplified by considering the case of mutants selected one dilution step below the MPC whose MIC is equal to or greater than its MPC value (Table 2).

Plasmid-mediated quinolone resistance enables mutant bacteria with low levels of resistance to survive long enough for them to grow and emerge during fluoroquinolone treatment. Interestingly, the MICs of some mutants from transconjugants containing *qnrA1* and deriving from *E. coli* J53 and *K. pneumoniae* C2/pMG252 were as high as 4 μg/ml and 32 to 128 μg/ml, respectively (Table 2). Primers used to detect mutations in *gyrA*, *parC*, and *qnrA1* have been described previously (8, 19). Mutations in the quinolone resistance-determining re-

* Corresponding author. Mailing address: Department of Microbiology, University of Seville, Sanchez Pizjuan s/n, Seville 41009, Spain. Phone: 34 954 55 28 63. Fax: 34 954 37 74 13. E-mail: jmrodriguez@us.es.

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TABLE 1. Quinolone susceptibility characteristics of strains included in this study

Strain	Description	CIP susceptibility ^a	Mechanisms associated with quinolone resistance				Source or reference
			<i>qnrA1</i> ^b	GyrA	ParC	Porins ^d	
UAB1	<i>K. pneumoniae</i> clinical strain	I	+	wt ^c	wt	+	11
N5	<i>K. pneumoniae</i> clinical strain	S	+	wt	wt	+	17
1960	<i>K. pneumoniae</i> clinical strain	S	+	Ser83Phe	wt	+	17
1132	<i>K. pneumoniae</i> clinical strain	R	+	wt	wt	+	17
DH10B	<i>E. coli</i> reference lab strain	S	-	wt	wt	+	
DH10B/pACYC184	<i>E. coli</i> DH10B containing pACYC184 vector	S	-	wt	wt	+	This study
DH10B+ <i>qnrA1</i>	<i>E. coli</i> DH10B containing <i>qnrA1</i> cloned in pACYC184 vector	S	+	wt	wt	+	This study
<i>E. coli</i> J53	<i>E. coli</i> reference lab strain	S	-	wt	wt	+	
J53/pMG252	<i>E. coli</i> J53 transconjugant derived from UAB1	S	+	wt	wt	+	11
J53/pN5	<i>E. coli</i> J53 transconjugant derived from N5	S	+	wt	wt	+	17
J53/p1960	<i>E. coli</i> J53 transconjugant derived from 1960	S	+	wt	wt	+	17
J53/p1132 ⁻	<i>E. coli</i> J53 transconjugant derived from 1132	S	-	wt	wt	+	17
C2	<i>K. pneumoniae</i> clinical strain	S	-	Ser83Phe	wt	-	11
C2/pMG252	C2 transconjugant derived from UAB1	I	+	Ser83Phe	wt	-	This study

^a S, susceptible, I, intermediate susceptibility, R, resistance according to CLSI guidelines (4).

^b +, Presence and expression of *qnrA1*; -, no expression.

^c wt, wild type.

^d + or - indicates the presence or absence of porins, respectively.

gions of *gyrA* or *parC*, which are related to the fluoroquinolone resistance in *Enterobacteriaceae*, were found in *qnrA1*-expressing mutants (Ser83Phe and Asp87Tyr in GyrA, and Ser80Ile and Val87Asp in ParC). No mutations were found either in the *qnrA1* coding sequences or in their promoter sequences (data not shown) (18). Additional mutations in *qnrA1*-containing strains relating to quinolone resistance and detected in the type II topoisomerase genes are a reflection of the ability of this mechanism to select for mutants with additional chromosomal quinolone resistance mechanisms. Such mechanisms, because of their additive nature, lead to therapeutic failure (10). In vivo selection of fluoroquinolone-resistant *E. coli* expressing the *qnrA1* gene was reported recently in the case of a patient with a urinary tract infection being treated with nor-

floxacin for an infection due to a ciprofloxacin-susceptible isolate (13). These data indicate the role of *qnrA1* in the selection and generation of quinolone-resistant mutants.

On the other hand, *qnrA1* greatly eased the mutant selection (Fig. 1). For example, in the presence of *qnrA1* (*E. coli* DH10B+*qnrA1*) and at 0.25 µg/ml, more than 10³ resistant colonies grew from an initial population of 10¹⁰ bacteria, and colonies could still be recovered at ciprofloxacin concentrations of 1 to 2 µg/ml (Fig. 1A). Expression of *qnrA1* from a natural plasmid in *E. coli* J53 produced over 10⁵ resistant colonies grown at 1 µg/ml for J53 pMG252, and colonies could still be recovered at moxifloxacin concentrations of 2 to 4 µg/ml (Fig. 1B). In the case of isogenic strains of *K. pneumoniae* C2, the expression of *qnrA1* from a natural plasmid

TABLE 2. Fluoroquinolone MPCs and MICs for clinical and isogenic strains used in this study and derived resistant mutants^a

Strain	MIC			MPC			MPC/MIC (MSW)			MIC for mutants ^b			MPC time (h) window ^c		
	CIP	LFX	MXF	CIP	LFX	MXF	CIP	LFX	MXF	CIP	LFX	MXF	CIP	LFX	MXF
UAB1	2	2	4	16	16	32	8	8	8	16-64	16-32	32-64	72-96	96	72
N5	0.25	0.5	0.5	4	4	16	16	8	32	8	8	4-8	48	48	24-48
1960	1	2	2	64	64	32	64	32	16	32-64	64	16	72	72	24-48
1132	4	4	8	32	32	32	8	8	4	16	16	16	48	48	72
<i>E. coli</i> DH10B	0.002	0.004	0.002	0.015	0.015	0.06	7.5	3.8	30	0.008	0.008	0.06	72	72	96
<i>E. coli</i> DH10B/pACYC184	0.002	0.004	0.002	0.015	0.015	0.06	7.5	3.8	30	0.008	0.008	0.06	72	72	72
<i>E. coli</i> DH10B+ <i>qnrA1</i>	0.125	0.125	0.125	2	2	2	16	16	16	1	1	0.5	72-96	96	96
<i>E. coli</i> J53	0.004	0.008	0.015	0.06	0.06	0.125	15	7.5	16.7	0.03	0.03	0.5	48	48	72
J53/pMG252	0.25	0.25	0.5	4	4	4	16	16	8	4	2	2-4	96	96	72
J53/pN5	0.125	0.125	0.125	4	4	4	32	32	32	2	4	2	72	96	72
J53/p1960	0.125	0.125	0.125	4	4	4	32	32	32	1	2	4	72	96	72
J53/p1132 ⁻	0.004	0.008	0.015	0.06	0.06	0.25	15	7.5	16.7	0.03	0.06	0.25	72	48	72
C2	0.25	0.25	0.125	8	8	4	32	32	32	4-8	8	2-8	24-48	48	24-48
C2/pMG252	2	2	1	128	128	32	64	64	32	64-128	64	32-64	24-48	72	24-48

^a MICs (in µg/ml) were determined by microdilution for ciprofloxacin (CIP), levofloxacin (LFX), or moxifloxacin (MXF). MPC values (in µg/ml) were determined for ciprofloxacin, levofloxacin, or moxifloxacin on Mueller-Hinton plates as the lowest antibiotic concentration (in the range of concentration steps analyzed) at which resistant colonies do not form. The MSW is the mutant selection window, the antibiotic concentration found between the MIC and the MPC.

^b MIC for CIP (microdilution) for ciprofloxacin- or moxifloxacin-resistant colonies were recovered on Mueller-Hinton plates one step below the MPC value.

^c Earliest time (in hours) at which resistant colonies were visible one step below the MPC.

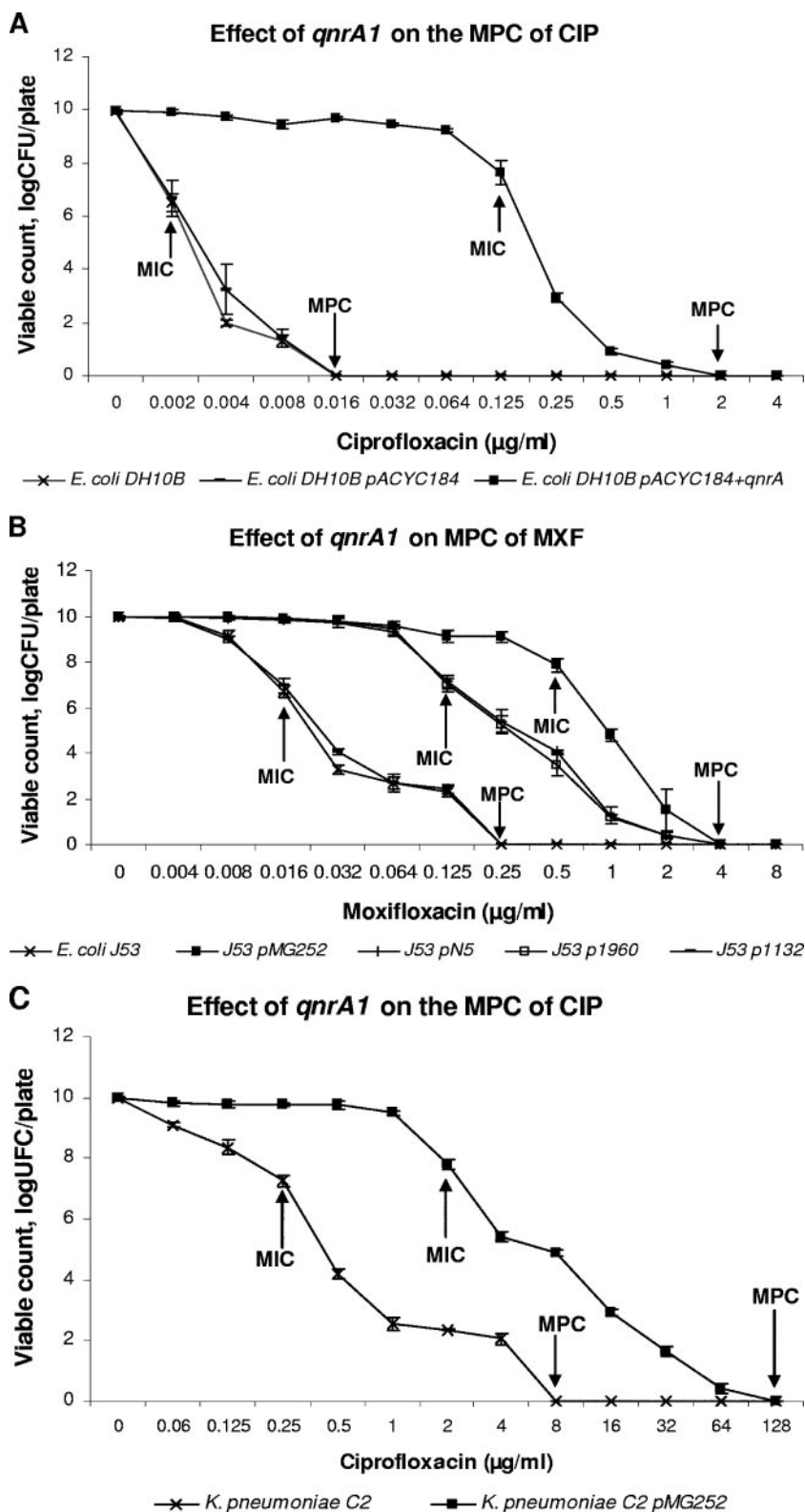


FIG. 1. MPC assays. MIC and MPC values are indicated on figures by arrows. CIP, ciprofloxacin; MXF, moxifloxacin.

produced more than 10^3 resistant colonies grown at 16 $\mu\text{g/ml}$, and colonies were still visualized at ciprofloxacin concentrations of 64 to 128 $\mu\text{g/ml}$ (Fig. 1C), levels higher than peak serum concentrations attained during drug therapy. In cases of

strains containing *qnrA1*, doses exceeding MPC in monotherapy would not be advisable with approved dosing procedures and evaluations of drug toxicity (2, 3). The increased frequency of quinolone-resistant mutants in strains which ex-

press *qnrA1* at different concentrations below the MPC provides support for the role of low-level resistance mediated by the mechanism in selecting for quinolone resistance (11, 15), particularly if we take into account the MICs observed in mutants selected below the MPC and include some mutants obtained from strains whose only quinolone resistance mechanism was *qnrA1* (Table 2).

Bacteria surviving at MPC were counted after 96 h, as described elsewhere (9). The MIC and MPC of these organisms (isolated at very low frequencies [data not shown]) were similar to those of the original strains. Although some bacteria survived MPC for a prolonged period, no quinolone-resistant mutants were selected, showing that the MPC parameter was working as specified. It is possible that survival is related to mutations in the *hip* genes (high persistence), being enhanced in the presence of β -lactams or fluoroquinolones, and producing a persistent phenotype at high antimicrobial concentrations (22).

Mechanisms, such as *qnr* or the recently described *aac(6')-Ib-cr* gene, which are implicated in low-level fluoroquinolone resistance to plasmid-mediated determinants, may play a significant role in the generation of resistant mutants and therapeutic failure. This may be because, first, many clinical strains reported as containing genes that code for plasmid-mediated fluoroquinolone resistance are susceptible and, second, the presence of such genes in conjugative plasmid or other mobile elements may facilitate rapid dissemination in *Enterobacteriaceae* and other bacteria of clinical significance. Animal models will be necessary in future to perform in vivo validation of these in vitro data obtained by using the MPC parameter.

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