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MECHANISMS OF RESISTANCE

Plasmidic *qnr* Genes Confer Clinical Resistance to Ciprofloxacin under Urinary Tract Physiological Conditions

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ABSTRACT *Escherichia coli* variants expressing plasmid-mediated *qnr* genes are usually susceptible to fluoroquinolones by standard susceptibility testing. Here we show that, under specific urinary tract physiological conditions, susceptible laboratory and clinical strains harboring *qnr* determinants become fully resistant to ciprofloxacin (CIP). Therefore, physiological conditions, mainly urine pH values, should be considered when performing susceptibility testing of CIP activity against *E. coli* in treating urinary tract infection (UTI) and for selecting appropriate antibiotics for UTI treatment.

KEYWORDS ciprofloxacin, *Escherichia coli*, MIC, pH, *qnr*, urinary tract infection

Received 10 December 2016 **Returned for modification** 4 January 2017 **Accepted** 9 January 2017

Accepted manuscript posted online 17 January 2017

Citation Martín-Gutiérrez G, Rodríguez-Martínez JM, Pascual Á, Rodríguez-Beltrán J, Blázquez J. 2017. Plasmidic *qnr* genes confer clinical resistance to ciprofloxacin under urinary tract physiological conditions. Antimicrob Agents Chemother 61:e02615-16. https://doi.org/10.1128/AAC.02615-16.

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Fluoroquinolones (FQs) are broad-spectrum antibiotics commonly used for urinary tract infection (UTI) (1–3). However, in recent years, a worrisome increase in resistance to FQs has been addressed worldwide (4, 5). Bacterial resistance to FQs is achieved mainly through the acquisition of sequential mutations in genes encoding DNA gyrase and topoisomerase IV (6, 7). The presence of some of these highly prevalent mutations (8, 9) often confers a low-level quinolone resistance (LLQR) phenotype with an MIC lower than the clinical breakpoint but higher than the epidemiological cutoff (10, 11). Therefore, low-level quinolone-resistant *Escherichia coli* mutants have been traditionally considered to be susceptible according to standard susceptibility testing. However, we recently found that growth under physiological urinary tract conditions (i.e., growth in urine, at low pH values, and under anaerobiosis) conferred clinical levels of resistance to the fluoroquinolone ciprofloxacin (CIP) (12). This situation may provide a selective scenario for the evolution of high-level quinolone resistance and could lead to therapeutic failure in patients with urinary tract infection (UTI).

In contrast, plasmid-mediated low-level resistance mechanisms may also compromise the efficacy of quinolones (13). The first report ofplasmid-mediated quinolone resistance described the presence of a 218-amino-acid protein termed Qnr that protects DNA from quinolone binding to topoisomerases (14). Since then, five groups of plasmidic Qnr determinants (encoded by the genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC*) have been described (15). Due to their plasmidic nature, *qnr* genes can be horizontally transferred, which confers an LLQR phenotype (15, 16). Furthermore, *qnr* genes are often associated with extended-spectrum β-lactamases and aminoglycoside-resistance-encoding genes on the same plasmid (17), mutually increasing the probability of dissemination (18).

TABLE 1 CIP MICs for low-level quinolone-resistant *E. coli* strains harboring *qnr* determinants in MHB and urine at different pH values

		CIP MIC (μg/ml) for[a]:							
		MHB (pH 7.4)		UR (pH 7)		UR (pH 6)		(UR pH 5)	
Strain	Genotype	O_2	nO_2	O_2	nO_2	O_2	nO_2	O_2	nO_2
Isogenic									
ATCC 25922	*pBK*-CMV	0.006	0.015	0.015	0.03	0.06	0.25	1	1
EC10	*pBK-qnrA1*	0.25	1	0.25	1	**4**	**4**	**32**	**32**
EC11	*pBK-qnrB1*	0.25	0.5	0.25	1	**4**	**4**	**32**	**32**
EC12	*pBK-qnrC*	0.125	0.5	0.25	1	**4**	**4**	**32**	**32**
EC13	*pBK-qnrD1*	0.06	0.06	0.03	0.25	0.5	1	**8**	**8**
EC14	*pBK-qnrS1*	0.125	0.5	0.25	2	**4**	**8**	**32**	**32**
UPEC									
PMQR 2	*qnrS1*	0.25	0.5	0.5	2	**8**	**8**	**64**	**64**
PMQR 11	*qnrB4*	0.25	0.5	0.5	1	**8**	**8**	**64**	**64**
PMQR 13	*qnrS1*	0.25	0.5	1	2	**8**	**8**	**128**	**64**
PMQR 30	*qnrB4*	0.125	0.5	0.25	0.5	**4**	**8**	**64**	**64**
PMQR 51	*qnrB4*	0.25	1	0.5	1	**8**	**8**	**64**	**64**
PMQR 73	*qnrA1*	0.25	0.5	0.5	0.5	**4**	**4**	**32**	**32**
PMQR 88	*qnrS1*	0.25	0.5	0.5	0.5	**4**	**4**	**64**	**32**
PMQR 91	*qnrS1*	0.25	0.5	0.5	**2**	**4**	**8**	**64**	**32**
PMQR 100	*qnrB4*	0.125	0.5	0.25	0.5	**4**	**4**	**32**	**64**

[a]Resistant values, according to CLSI guidelines, are shown in bold. MHB, Mueller-Hinton broth; UR, urine; O_2, aerobic conditions; nO_2, anaerobic conditions; UPEC, uropathogenic *E. coli*.

The prevalence of *qnr* genes in clinical urinary isolates has ranged from 10.81% to 31.6% (19, 20). This prevalence has varied depending on geographical location, patient characteristics (e.g., higher prevalence in inpatients than outpatients [20]), and associated susceptibility patterns (21, 22). However, the causes of this moderately high prevalence among UTI isolates remain puzzling, as Qnr determinants confer only a slight increase in resistance (below the clinical breakpoint) that should not be enough to withstand the extremely high quinolone concentrations attained in the urinary tract.

In view of the foregoing facts, we decided to study the activity of CIP against a set of well-characterized *qnr*-expressing strains under simulated urinary tract physiological conditions. For this purpose, we studied the CIP susceptibilities of five *E. coli* ATCC 25922 derivatives harboring the *qnrA1*, *qnrB1*, *qnrC*, *qnrD1*, and *qnrS1* genes cloned into the pBK-CMV plasmid (23), the control strain harboring the empty vector lacking any *qnr* gene, and nine *E. coli* clinical strains harboring well-characterized Qnr determinants (24) (Table 1). These strains had no mutations in their *gyrA* or *parC* genes, as demonstrated by sequencing analysis (24). Susceptibility to CIP was determined by the microdilution method using Mueller-Hinton broth (MHB) following Clinical and Laboratory Standards Institute (CLSI) recommendations (25). To reproduce UTI physiological conditions (urine, different pH values, and anaerobiosis), MICs in urine were also determined at pH values of 5, 6, and 7, and plates were incubated under aerobic conditions and under anaerobic conditions. Urine was obtained as previously described (12). MICs of CIP were determined in triplicate for each strain. Results were interpreted following CLSI clinical breakpoints for CIP (susceptible, $\leq$1 μg/ml; intermediate, 2 μg/ml; resistant $\geq$4 μg/ml).

As shown in Table 1, pH, urine, and anaerobiosis each had a great effect on CIP activity against the *qnr*-expressing strains. At neutral pH, growth in urine increased the MICs from 2- to 4-fold over the growth in MHB. We observed an increase in MICs from 4- to 16-fold when urine pH values decreased from 7 to 6. This implies that all the Qnr strains (both clinical and ATCC derivatives), except the one harboring *qnrD1*, became resistant in urine at pH 6. At pH 5, all the Qnr strains became resistant, showing increases in MIC values ranging from 16- to 128-fold. Growth under anaerobic conditions showed mild increases in MICs, ranging from 2- to 8-fold, depending on the

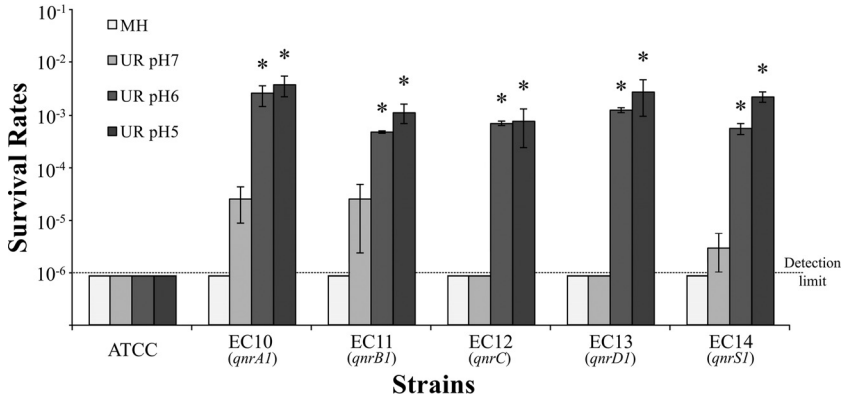

FIG 1 Survival rates of *E. coli* carrying *qnr* determinants after 6 h of treatment with 1,000 μg/ml of CIP under anaerobic conditions. Survival rates were calculated by normalizing the number of surviving bacteria to the estimated initial population. Error bars represent standard deviations. Asterisks denote statistically significant differences between conditions compared with survival rate in Mueller-Hinton broth for each strain ($P < 0.05$, Student's *t* test). The pH of MH was 7.4.

conditions. Notably, similar increases in resistance were observed for the clinical strains studied. These results demonstrate that urinary tract physiological conditions considerably affect the susceptibility of the isogenic and clinical Qnr strains, making all of them resistant to CIP in urine at pH 5. It should be noted here that the urine of more than 60% of the UTI patients had a pH of $\leq$6 (12).

With the aim of testing how survival of Qnr strains under CIP treatment could be affected by urinary tract physiological conditions, we also determined the survival rates of isogenic strains in the presence of the maximum concentration of CIP attained in the bladder ($\sim$1,000 μg/ml) during the first 6 h of treatment, according to previous pharmacokinetic and pharmacodynamic (PK/PD) studies (26). About 5×10^5 bacterial cells/ml were inoculated into MHB or urine at different pH values containing 1,000 μg/ml of CIP, and samples were incubated at 37°C for 6 h under anaerobic conditions. Each strain was evaluated in triplicate. As can be seen in Fig. 1, when grown in urine at pH 6 or 5, survival rates of each of the Qnr strains increased from 3 to 4 orders of magnitude compared with those of the same strains at pH 7 ($P < 0.05$, Student's *t* test).

The results of this work show that urinary tract physiological conditions can create an ideal environment for the selection of strains harboring *qnr* genes, decreasing their susceptibility to CIP and allowing the survival of microorganisms traditionally considered susceptible. In this way, the presence of physiological UTI conditions combined with the effectiveness of *qnr* horizontal transfer and the coselection with other resistance genes might play a critical role in the rapid spread of multidrug resistance throughout uropathogenic *E. coli* strains. Under this assumption, international guidelines for MIC determinations should prompt a reconsideration of clinical breakpoints, taking into account pH values for CIP activity. Furthermore, urine pH values should be considered by physicians to be an important aspect when selecting an antibiotic for UTI treatment.

ACKNOWLEDGMENTS

J.B. was supported by the Subdirección General de Redes y Centros de Investigación Cooperativa, Ministerio de Economía y Competitividad, Instituto de Salud Carlos III, Spanish Network for Research in Infectious Diseases (grants RD12/0015/0012 and REIPI RD12/0015/0012), cofinanced by European Development Regional Fund "A way to achieve Europe," and Fondo de Investigación Sanitaria (grant PI13/00063). J.M.R.-M. was supported by Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía (grant P11-CTS-7730).

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