AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy

Urinary Tract Physiological Conditions Promote Ciprofloxacin Resistance in Low-Level-Quinolone-Resistant *Escherichia coli*

Guillermo Martín-Gutiérrez,[a,c] Jerónimo Rodríguez-Beltrán,[a] José Manuel Rodríguez-Martínez,[b] Coloma Costas,[a] Javier Aznar,[a,c] Álvaro Pascual,[b,c] Jesús Blázquez[a,d]

Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío, CSIC, Universidad de Sevilla, Seville, Spain[a]; Departamento de Microbiología, Universidad de Sevilla, Seville, Spain[b]; Unidad Intercentros de Enfermedades Infecciosas, Microbiología y Medicina Preventiva, Hospitales Universitarios Virgen Macarena y Virgen del Rocío, Seville, Spain[c]; Centro Nacional de Biotecnología (CNB), Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain[d]

***Escherichia coli* isolates carrying chromosomally encoded low-level-quinolone-resistant (LLQR) determinants are frequently found in urinary tract infections (UTIs). LLQR mutations are considered the first step in the evolutionary pathway producing high-level fluoroquinolone resistance. Therefore, their evolution and dissemination might influence the outcome of fluoroquinolone treatments of UTI. Previous studies support the notion that low urine pH decreases susceptibility to ciprofloxacin (CIP) in *E. coli*. However, the effect of the urinary tract physiological parameters on the activity of ciprofloxacin against LLQR *E. coli* strains has received little attention. We have studied the activity of ciprofloxacin under physiological urinary tract conditions against a set of well-characterized isogenic *E. coli* derivatives carrying the most prevalent chromosomal mutations (Δ*marR*, *gyrA*-S83L, *gyrA*-D87N, and *parC*-S80R and some combinations). The results presented here demonstrate that all the LLQR strains studied became resistant to ciprofloxacin (according to CLSI guidelines) under physiological conditions whereas the control strain lacking LLQR mutations did not. Moreover, the survival of some LLQR *E. coli* variants increased up to 100-fold after challenge with a high concentration of ciprofloxacin under UTI conditions compared to the results seen with Mueller-Hinton broth. These selective conditions could explain the high prevalence of LLQR mutations in *E. coli*. Furthermore, our data strongly suggest that recommended methods for MIC determination produce poor estimations of CIP activity against LLQR *E. coli* in UTIs.**

Ciprofloxacin (CIP) is one of the agents commonly utilized for treatment of urinary tract infections (UTIs) (1–3). It is used as an appropriate therapy in patients with UTI not requiring hospitalization in areas where the prevalence of resistance is under 10%. In addition, it is considered an effective treatment in the prevention of UTI in kidney transplant recipients (4, 5). However, during recent years a clear increase in resistance to fluoroquinolones (FQ) has been described worldwide (6, 7).

The predominant causative agent of community-acquired UTI, *Escherichia coli*, acquires resistance mainly through chromosomal mutations in the genes encoding subunits of the DNA gyrase (*gyrA* and *gyrB* genes) and topoisomerase IV (*parC* and *parE* genes) or in regulatory genes affecting permeability or efflux (8). Some of these mutations lead to generation of low-level-quinolone-resistant (LLQR) *E. coli* mutants, with a CIP MIC that is higher than the epidemiological cutoff value but still below the resistance breakpoint for most FQs. A high (from 17% to 39%, depending on the study) prevalence of LLQR *E. coli* in UTIs has been previously described (8, 9). However, the causes of this prevalence are unclear, as the high CIP levels found in the bladder after treatment should be sufficient to eradicate any susceptible bacteria.

It has been stated that small increases in MIC values produced by LLQR mutations have a profound impact on the mutant, preventing concentration of FQs and raising the likelihood that strains with high-level resistance will appear (10). Therefore, LLQR mutants are arguably the first step on the evolutionary pathway to producing high-level quinolone resistance, and thus their dissemination and evolution might influence the outcome of FQ treatment of UTI.

One of the determinants of this outcome is the environment found in the urinary tract, which is characterized mainly by the presence of urine and anaerobic conditions. Urine is a fluctuating and complex fluid composed of over 95% water, plus sodium, ammonia, phosphate, sulfate, urea, creatinine, proteins, and products processed by the kidney and liver (11). The variability in the physicochemical properties of urine is best illustrated by the wide pH range of human urine, which normally varies from 5 to 8 (12). Additionally, the bladder environment is mainly anaerobic, with a concentration of dissolved oxygen (DO) in urine of about 4.2 ppm (13); the concentration is also variable and mainly reflects the renal metabolic state. Moreover, in patients with urinary infections, the urine DO concentration is significantly reduced as a result of oxygen consumption by the infecting microbes (13).

However, the relative contributions of these parameters to the possible outcomes of CIP treatment against LLQR strains have received little attention. CIP is an ampholyte consisting of an acidic group and a basic group that can exist in four different pH-dependent protonation forms (14). The zwitterionic form is necessary for bacterial permeability, while the neutral form provides good intestinal absorption (15). Therefore, the wide range of

Received 16 March 2016 **Returned for modification** 24 April 2016
Accepted 27 April 2016
Accepted manuscript posted online 2 May 2016
Citation Martín-Gutiérrez G, Rodríguez-Beltrán J, Rodríguez-Martínez JM, Costas C, Aznar J, Pascual A, Blázquez J. 2016. Urinary tract physiological conditions promote ciprofloxacin resistance in low-level-quinolone-resistant *Escherichia coli*. Antimicrob Agents Chemother 60:4252–4258. doi:10.1128/AAC.00602-16.
Address correspondence to Jesús Blázquez, blazquez@cnb.csic.es.
G.M.-G. and J.R.-B. contributed equally to this article.

TABLE 1 Genotypes of isogenic and UPEC strains[a]

Strain	Genotype			
	gyrA1	*gyrA2*	*parC*	*marR*
Isogenic				
ATCC 25922				
EC01				Δ
EC02	S83L			
EC03	S83L			Δ
EC04	S83L		S80R	
EC05	S83L		S80R	Δ
EC06	S83L	D87N		
EC07	S83L	D87N		Δ
EC08	S83L	D87N	S80R	
EC09	S83L	D87N	S80R	Δ
UPEC				
Val09	S83L			
MAC02	S83L			
MAC10	S83L			
MAC11	S83L		S80R	
MAC21a	S83L		S80I	
MAC33		D87Y		

[a] UPEC, uropathogenic *Escherichia coli* strains; Δ, precise deletion.

pH found in human urine can affect the ionization status of CIP and can thus modify its antimicrobial activity (16–19). Regarding oxygen levels, it has been reported that growth under conditions of anaerobiosis affects bacterial susceptibility to CIP (20). Therefore, both parameters could, in principle, influence the resistance profiles of LLQR UTI strains, thus affecting the prognosis of treatment.

Pharmacokinetic and pharmacodynamic (PK/PD) studies indicate that very high (up to 990 μg/ml) concentrations of CIP can be reached in urine during treatment (21), suggesting that, despite a possible effect of pH and other urine parameters on CIP activity, such treatment should be sufficient to kill any susceptible bacteria. However, to date, no studies on the effect of these parameters on the activity of CIP against strains containing LLQR mutations have been performed.

Consequently, we aimed to evaluate the impact of the above-mentioned parameters (namely, growth in urine, pH, and anaerobiosis) on the antimicrobial activity of CIP against a set of well-characterized isogenic LLQR strains carrying the most frequent chromosomal mutations (9, 22) and against a series of *E. coli* clinical isolates with known LLQR mutations. MICs of CIP in both Mueller-Hinton broth (MH) and urine under various pH and oxygen supply conditions were studied. In addition, the survival rates of these strains in the presence of the maximum concentration of CIP reached in the bladder were also analyzed.

MATERIALS AND METHODS

Strains and growth conditions. Wild-type *E. coli* ATCC 25922 and nine different isogenic strains carrying combinations of the most prevalent chromosomal mutations (Δ*marR*, *gyrA*-S83L, *gyrA*-D87N, and *parC*-S80R) were used. Isogenic strains were those constructed previously by Machuca et al. (23). Moreover, six uropathogenic *E. coli* (UPEC) strains with well-characterized LLQR mutations that had been isolated from patients in the University Hospital Marques de Valdecilla and University Hospital Virgen Macarena during 2009 were studied (Table 1).

MH (Conda Pronadisa, Spain) was used as a control medium. For growth rate determinations, overnight cultures of the strains were diluted 1:100 into MH and cultured with aeration at 37°C during 2 h at 180 rpm to obtain bacteria in the exponential-growth phase. Growth assays were conducted in clear flat-bottom 96-well MicroWell plates (Nunc, Roskilde, Denmark), containing 8 replicates of 100 μl per sample. The plates were incubated at 37°C with shaking on an automated microplate reader (Infinite M200; Tecan, Männedorf, Switzerland), and the absorbance at 595 nm for each well was measured every 30 min. The duration of each assay was 24 h.

Urine obtained from 3 healthy volunteers who had not received antibiotic treatment during the previous 6 months was pooled, sterilized by filtration through 0.22-mm-pore-size filters (polyethersulfone [PES] membrane) (VWR, United Kingdom), divided into aliquots, and stored at −20°C. The urine was adjusted to pH values of 5.0, 6.0, and 7.0 with HCl or NaOH (both from Sigma-Aldrich), and the samples were again subjected to sterile filtration before use. Urine-agar plates were made by mixing 4:1 pH-adjusted pooled human urine with 6% agar (Conda Pronadisa, Spain). Final pH values were determined for urine-agar before plating and were readjusted when necessary.

Measurement of pH of urine from patients with community-acquired *E. coli* UTI. From March 2015 to June 2015, ≥18-year-old patients with UTI symptoms receiving care at the Primary Care Units of Virgen del Rocío University Hospital were included in this study. Midstream catch urine was collected in sterile preservative tubes (SRO-1-25B; Soria Melguizo S.A., Madrid, Spain) and was transported on ice. Urine samples were processed within 4 to 8 h after collection. For each sample, 10 μl of urine was quantitatively cultured onto Brilliance UTI Clarity agar plates (Oxoid, Basingstoke, United Kingdom). All plates were aerobically incubated for 18 to 24 h at 37°C, and the results were expressed as the number of CFU per milliliter. Urine pH was measured in each collected specimen with a pH meter.

Cultures in which *E. coli* levels were above thresholds of $\geq 10^5$ CFU/ml for women and $\geq 10^3$ CFU/ml for men were defined as positive. In total, pH values of urine from 136 patients (with proven UTI caused by *E. coli*) were included.

Susceptibility testing. MICs of CIP were determined in triplicate for each bacterial strain using the broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI) reference methods (24). Overnight cultures were prepared and adjusted to a density of 0.5 McFarland. To reproduce urine physiological conditions, the MICs of CIP were measured in MH and urine at pH values of 5, 6, and 7. To determine if the effect was due only to the decrease in pH, MICs were also measured in MH at pH 5 and pH 6, in addition to the standard pH of MH broth (7.3 ± 0.1). Microplates were incubated under aerobic and anaerobic conditions.

MICs of CIP were also determined by a gradient MIC strip methodology (Liofilchem srl, Italy) according to CLSI criteria. Bacterial suspensions were prepared from overnight cultures and adjusted to a density of 0.5 McFarland. Gradient MIC strip experiments were performed on MH agar (Difco, Soria Melguizo, Spain) and urine-agar with pH values of 5, 6, and 7. Plates were incubated under aerobic conditions for 24 h (48 h for urine-agar tests) at 37°C.

Survival rates. Overnight cultures of the different strains were grown at 37°C without the antimicrobial agent in both MH and urine at different pH values. Then, bacteria at a density of $\sim 5 \times 10^5$ CFU/ml from these cultures were inoculated into fresh MH or urine containing CIP and were incubated at 37°C for 6 h in anaerobiosis. The quinolone concentration used for determination of survival rates was 1,000 μg/ml, slightly above the maximum concentration of CIP reached in urine within the first 6 h after administration of an oral dose of 500 mg CIP (21), which is the recommended dose for adults (4). Antibiotic-free broth was evaluated in parallel as a control. Viable counts were determined by plating serial dilutions onto MH agar plates incubated at 37°C for up to 24 h. The survival rate was calculated by normalizing the number of surviving bacteria to the estimated initial population.

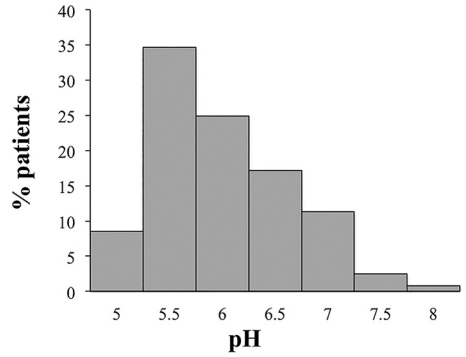

FIG 1 Distribution of urine pH values in 136 samples from patients with UTI caused by *E. coli*.

RESULTS

Variability of pH in urine from UTI patients. We first conducted a prospective study to measure the urine pH from patients with community-acquired *E. coli* UTI. The results presented in Fig. 1 demonstrate a wide variability in pH values, ranging from 5 to 8, suggesting that physiological urinary pH is highly variable among patients. We found that the vast majority (90%) of pH values were below neutrality, with most samples being markedly acidic (63% with pH = ≤6). This result, which agrees with a previous report (25), indicates that low urine pH is the rule rather than the exception in UTI patients.

Effect of urine pH on the growth of *E. coli* ATCC 25922. We then studied the ability of the *E. coli* ATCC 25922 strain to grow in urine at physiological pH values. Growth curves in MH and urine were carried out using an automated spectrophotometer under controlled conditions of temperature and shaking. Figure 2 shows that *E. coli* ATCC 25922 was able to prosper in urine, although its growth was somewhat lower than that in MH, probably due to the low concentration of nutrients. Acidification of urine, including pH 5, led to only small decreases in growth, demonstrating that *E. coli* is well adapted to extreme pH values. Similarly, all LLQR derivatives were able to grow in urine at different pH values (data not shown). It should be noted here that we were unable to perform growth curve determinations in basic urine (pH 8) because, under our conditions, urine components precipitate at pH values higher than 7. This, together with the fact that basic urine is uncommon among UTI patients (<5%) (Fig. 1), led us to exclude this condition from further experiments.

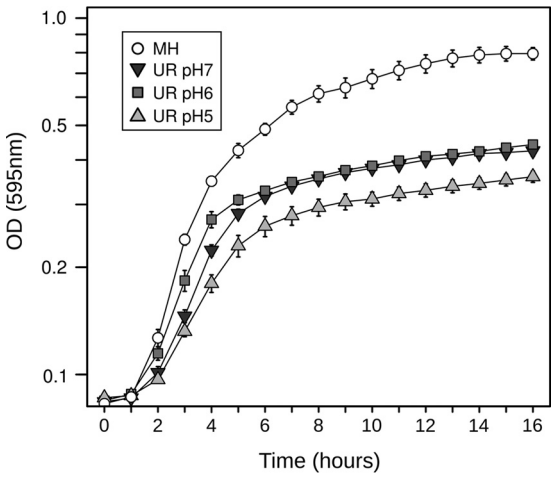

FIG 2 Growth curves of *E. coli* ATCC 25922 growing in MH and urine at different pH values. Error bars represent standard errors of the means of results from at least four replicates. MH, Mueller-Hinton; UR, urine; OD, optical density.

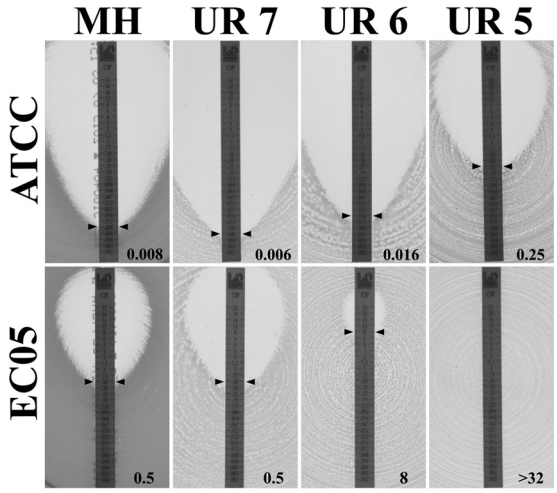

FIG 3 CIP susceptibility testing for two representative strains (ATCC 25922 and EC05), using the gradient MIC strip methodology. It was observed that pH has a great influence on CIP activity, with a significant increase in MICs when pH decreases. MICs (shown in micrograms per milliliter) are indicated at the right bottom for each condition. UR 7, urine-agar pH 7; UR 6, urine-agar pH 6; UR 5, urine-agar pH 5.

Effect of pH and anaerobiosis on the activity of CIP against LLQR *E. coli* strains growing in urine. To determine if CIP activity is affected by urine acidification, we performed preliminary MIC determinations using of a combination of the gradient MIC strip methodology and pH-adjusted urine agar plates (see Materials and Methods). As expected, pH values had a large influence on the CIP MICs detected by gradient MIC strips for all LLQR strains. Figure 3 shows an illustrative example with ATCC 25922 and its *gyrA*-S83L–*parC*-S80R derivative EC05.

The activity of CIP against all LLQR isogenic derivatives was also determined in urine under different conditions (pH and anaerobiosis) by the microdilution method. Table 2 confirms that pH had a large influence on MICs, with 4-fold to 16-fold MIC increases in the ATCC 25922 strain and its isogenic LLQR derivatives when the pH was changed from neutral to 6. Importantly, with the exception of the ATCC 25922 and EC01 (Δ*marR*) strains, all susceptible strains became resistant (MIC of >4 μg/ml) according to CLSI classification. The MIC increases were even higher when the urine pH was set to 5. Under those conditions, CIP activity decreased 32-fold to 256-fold in comparison to the activity seen at pH 7. At pH 5, all strains except ATCC 25922 were resistant to CIP, as defined by CLSI guidelines. Taken together, these results indicate that the presence of any of the studied LLQR mutations was enough to confer clinical levels of resistance at acidic but physiological pH. Remarkably, the ATCC 25922 strain, which lacks any of these resistance determinants, remained susceptible under these conditions.

Regarding the effect of anaerobiosis, Table 2 (nO_2 columns) shows that growth under anaerobic conditions increased the CIP MICs 2-fold to 8-fold at pH 7 compared to the values obtained

TABLE 2 Ciprofloxacin MICs for *E. coli* strains in urine at different pH values[a]

	MIC (μg/ml)					
	UR pH 7		UR pH 6		UR pH 5	
Strain	O_2	nO_2	O_2	nO_2	O_2	nO_2
ATCC 25922	0.015	0.03	0.06	0.25	1	1
EC01	0.03	0.06	0.25	0.5	**4**	**2**
EC02	0.25	1	**4**	**4**	**32**	**32**
EC03	0.5	**2**	**8**	**16**	**64**	**32**
EC04	0.5	**2**	**8**	**16**	**64**	**64**
EC05	1	**4**	**16**	**64**	**256**	**256**
EC06	1	1	**4**	**4**	**32**	**32**
EC07	0.5	**2**	**8**	**16**	**64**	**64**
EC08	**4**	**8**	**32**	**64**	**512**	**512**
EC09	**8**	**16**	**64**	**256**	**512**	**512**

[a] Intermediate and resistant MIC values according to the CLSI guideline are shown in bold. O_2, aerobic incubation; nO_2, anaerobic incubation.

TABLE 4 Mean fold changes in MICs of ciprofloxacin in MH broth and urine at different values of pH and anaerobiosis[a]

	MIC (μg/ml) in MH broth and urine at pH:					
	7		6		5	
Strain	O_2	nO_2	O_2	nO_2	O_2	nO_2
ATCC 25922	4	8	32	64	128	256
EC01	1	8	8	64	32	256
EC02	4	8	16	32	128	256
EC03	2	8	16	64	32	128
EC04	2	4	16	32	64	128
EC05	1	4	16	64	64	256
EC06	4	8	16	32	128	256
EC07	2	8	16	64	64	256
EC08	2	4	16	32	128	256
EC09	1	4	16	64	32	128

[a] O_2, aerobic incubation; nO_2, anaerobic incubation.

with oxygen, converting all strains, except ATCC 25922 and EC01, to intermediate or resistant. The increases due to the absence of oxygen at pH 6 were minimal (from 2-fold to 4-fold), and there were almost no changes at pH 5.

In summary, these results demonstrate that the pH of urine had a great effect on the activity of CIP against the *E. coli* ATCC 25922 LLQR isogenic derivatives, with a pH of 5 capable of converting all LLQR strains to intermediate or resistant to CIP according to CLSI breakpoints. Furthermore, growth in urine at neutral pH under anaerobic conditions produced an increase in MIC values sufficient for classifying eight strains as resistant which would have been considered susceptible by traditional MIC determinations in MH.

Table 3 shows that, similarly to the results obtained with urine, acidification of MH had a great effect on CIP activity, with increases of 8-fold to 16-fold and 32-fold to 64-fold at pH 6 and 5, respectively. At pH 6, all strains, except ATCC 25922 and EC01, became resistant or intermediate, as defined by the CLSI clinical breakpoints. On the other hand, growth in the absence of oxygen had a smaller effect in MH than in urine, with CIP activity increases of 2-fold to 4-fold at pH 7 and almost no effect at pH 6 and 5. Therefore, growth in urine exacerbated the effect of pH and anaerobiosis on the CIP activity with respect to growth in MH.

MIC determinations under physiological conditions. Current methods employed to determine MICs in clinical microbiology laboratories are based on growth in MH broth or on MH agar plates in which the pH is close to neutrality (standard pH is 7.3 ± 0.1). Therefore, according to the results described above, when CIP reaches the bladder, the environmental conditions could alter its antimicrobial activity. Table 4 shows the fold change in MICs when the strains are grown in urine at different pH and oxygen levels in comparison to those obtained using classical MIC determination methods (MH broth, pH 7.3, and aerobiosis). These results indicate that anaerobic growth in urine increased the CIP MIC at pH 7 (2-fold to 8-fold), pH 6 (16-fold to 64-fold), and pH 5 (128-fold to 512-fold).

Survival rates at the maximum physiological CIP concentration. The results presented above suggest that CIP treatment of LLQR strains could be inefficient under UTI physiological conditions. To test this possibility, overnight cultures of the different strains were grown at 37°C without the antimicrobial agent in both MH and urine at different pH values. Then, bacteria at a density of about 5×10^5 CFU/ml were inoculated into fresh MH or urine containing CIP (1,000 μg/ml) and grown at 37°C during 6 h. The rationale for this experimental design was to mimic the best-case scenario, using the addition of the highest CIP concentration ever reported within the first 6 h after an oral dose of 500

TABLE 3 MICs of ciprofloxacin for isogenic *E. coli* strains in MH broth at different pH values[a]

	MIC (μg/ml) in MH broth at pH:					
	7		6		5	
Strain	O_2	nO_2	O_2	nO_2	O_2	nO_2
ATCC 25922	0.004	0.008	0.03	0.06	0.125	0.125
EC01	0.008	0.06	0.125	0.125	0.5	0.25
EC02	0.125	0.25	1	1	**4**	**4**
EC03	0.25	1	**2**	**4**	**8**	**8**
EC04	0.5	1	**4**	**4**	**16**	**16**
EC05	1	**4**	**8**	**8**	**64**	**32**
EC06	0.125	0.25	1	1	**4**	**4**
EC07	0.25	1	**4**	**4**	**16**	**16**
EC08	**2**	**4**	**16**	**16**	**64**	**128**
EC09	**4**	**16**	**32**	**64**	**256**	**128**

[a] Intermediate and resistant MIC values according to the CLSI guideline are shown in bold. O_2, aerobic incubation; nO_2, anaerobic incubation.

TABLE 5 Ciprofloxacin MICs against clinical strains in MH broth and urine at different pH values[a]

	MIC (μg/ml)							
	MH broth		Urine pH 7		Urine pH 6		Urine pH 5	
Strain	O_2	nO_2	O_2	nO_2	O_2	nO_2	O_2	nO_2
Val09	1	**2**	1	**8**	**8**	**16**	**256**	**256**
MAC02	0.125	0.25	0.25	0.5	1	1	**32**	**16**
MAC10	0.25	0.5	0.125	0.5	1	**4**	**32**	**16**
MAC11	0.5	**2**	0.25	**2**	**2**	**4**	**64**	**64**
MAC21a	0.5	**2**	0.25	**2**	**2**	**4**	**64**	**64**
MAC33	0.125	0.5	0.25	0.25	0.5	1	**8**	**8**

[a] Intermediate and resistant MIC values according to the CLSI guideline are shown in bold. O_2, aerobic incubation; nO_2, anaerobic incubation.

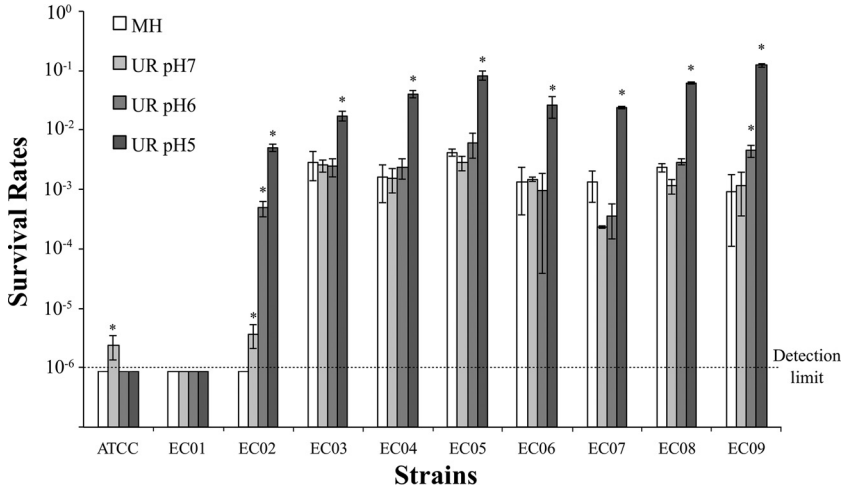

FIG 4 Survival rates of *E. coli* isogenic strains treated with 1,000 μg/ml of CIP after 6 h under anaerobic conditions. Survival rates were calculated by dividing the number of CFU per milliliter after CIP incubation by the value obtained at time zero. All results were obtained by at least three independent experiments. Error bars represent standard deviations. Asterisks denote statistically significant differences ($P < 0.05$ [Student's *t* test]) between conditions in comparison to the respective survival rate in Mueller-Hinton for every strain.

mg, although the concentration that CIP reaches in the bladder is typically lower (21). Note that we decided to use such a high concentration because we anticipated that the median CIP concentration during the first hours of treatment (268 μg/ml) (21) would be in a range near or below the MIC for several LLQR strains in urine under conditions of low pH (see Table 2); hence, the effects of CIP treatment against these strains would most likely be innocuous. Figure 4 shows that the survival rates of all strains, except ATCC 25922 and EC01, whose survival rates fall below the detection limit of our assay, increased substantially (10-fold to 100-fold) in urine at pH 5 compared with those seen with MH. At higher pH values, no statistically significant differences in survival were found, except for EC02.

Effect of urine, pH, and anaerobiosis on CIP activity against LLQR clinical isolates. To discard the possibility that the observed effects were specific to the *E. coli* ATCC 25922 strain and its derivatives, we scored the MICs of CIP against six well-characterized LLQR *E. coli* clinical strains. As for the ATCC 25922 strain, the MIC showed a marked increase when determinations were performed in urine at different pHs and under anaerobic conditions. In this case, three of six strains became intermediate, as defined by the CLSI breakpoints, when MIC determinations were performed in urine at pH 7 under conditions of anaerobiosis. Further increases were displayed in urine with acidic pHs; all clinical strains demonstrated resistance at pH 5 (Table 5).

DISCUSSION

The effectiveness of CIP for the treatment of UTI does not depend on pathogen susceptibility alone. Physicochemical and pharmacological properties of antibiotics are important factors related to successful therapy. Because CIP is an ampholyte (14), the pH of urine affects its ionization status, which is critical for its activity and cellular accumulation in both bacteria and bladder cells. Results of previous studies support the notion that low urine pH decreases susceptibility to CIP in a number of bacterial pathogens (16–19, 25).

Our results confirm that growth in urine at neutral pH slightly increases resistance to CIP in *E. coli* (26). This increase is further enhanced in an anaerobic environment, such as that found in the bladder. Importantly, growth in acidic urine renders all LLQR strains resistant to CIP according to CLSI breakpoints. These results suggest that traditional susceptibility testing in MH broth may result in poor estimations of CIP activity in UTI caused by LLQR strains.

It has been suggested that the high concentrations of FQ attained in the urinary tract are enough to eradicate bacterial pathogens, even those which, following CLSI guidelines, would have been considered resistant (27). This assumption is made based on the empirical success of treatments with CIP against resistant clones and the predictive values of PK/PD modeling for FQ effectiveness, which, given the high values of maximum concentration of drug in serum (C_{max}) and area under the concentration-time curve (AUC) reached in the urinary tract, predict the clinical response for strains with drug MIC values as high as ~20 μg/ml (28). According to our results, the application of this criterion to MIC data for LLQR strains suggests that growth in human urine, under physiological conditions of pH and oxygen concentration, could cause treatment failure in most cases. Similar behavior is expected for other fluoroquinolones; for instance, the activity of levofloxacin, moxifloxacin, and ofloxacin is also affected by growth in urine, pH, and anaerobiosis (29, 30).

To further validate this prediction, we developed a survival assay in which bacteria were exposed to an extremely high concentration of CIP (approximately 4-fold the median concentration in urine) within the first 6 h (21) in urine adjusted to different pH values and in anaerobiosis. Our results show that, especially under acidic conditions, survival is enhanced 10-fold to 100-fold in most strains compared to the rate seen with MH at neutral pH. Furthermore, most LLQR strains showed a relatively small (1 to 2 log) decrease in viability, despite the high CIP concentration that was used. In contrast, the strain lacking any FQ resistance determinant (ATCC 25922) was unable to cope with the simulated CIP treatment, highlighting the importance of LLQR mutations in treatment failure. Once again, these results raise issues regarding

the clinical success of CIP against LLQR strains under physiological conditions and should be interpreted as cautionary.

Despite the general belief that multiple mutations are required to generate clinically important resistance in *E. coli* (31), the results presented here show that UTIs could produce ideal conditions for the generation and selection of single LLQR mutants. Furthermore, the high survival rates that we found upon CIP exposure could be a determining factor in the development of multistep resistance mutants. FQs have been demonstrated to act as mutagens and to have a stimulatory effect on recombination frequency (32, 33), accelerating bacterial evolution and antibiotic resistance. It is conceivable, therefore, that in urine at low pH, prevalent mutations conferring LLQR such as GyrA S83L could provide the basal level of resistance needed to withstand the first hours of treatment. This may provide a window for the emergence of further resistance fueled by the mutagenic effect of CIP. In agreement, it has been reported that patients previously treated with FQ are prone to urinary tract infection caused by CIP-resistant *E. coli* (34). In summary, urinary tract conditions may represent an environment in which LLQR mutants would be selected under conditions of treatment with CIP (and possibly other FQs). This could explain, at least partially, the high prevalence of these mutations and raises concerns about the appropriateness of CIP treatments when LLQR strains are detected.

Concerning the clinical and physiological characteristics of patients with UTI caused by *E. coli*, there are several factors that should be taken into consideration when CIP is employed. It has been reported that the composition of the diet affects the acid-base balance in the body. A higher protein intake significantly increases renal acid excretion by increasing ammonium output, thus acidifying the urine (35, 36). Moreover, the effect of urine pH on CIP activity may become relevant in patients with certain underlying diseases. Thus, it is known that patients with hypertension are associated with a lower urinary citrate level and a higher level of acid excretion, resulting in lower urine pH values (37). This effect can also be found in patients with type 2 diabetes or metabolic acidosis (38, 39). There are some patient characteristics that are related to lower pH values in urine, such as older age or higher body weight (38, 40, 41). Some medications, such as loop diuretics or mineralocorticoids, can also acidify urine (42, 43). Therefore, treatment with CIP for UTI in patients with factors related to urine acidification could result in therapeutic failure and increased selective pressure, promoting the development of bacterial resistance. Thus, urinary pH values could have practical interest in the management of these patients, where the physician should select the course best suited to the individual patient: either basifying the urine by alkalinization (e.g., by the use of potassium citrate or sodium bicarbonate) (44, 45) with the aim of increasing the activity of CIP or electing to use other antibiotics not affected by low pH values.

ACKNOWLEDGMENT

We are grateful to Michael McConnell for useful scientific comments and English revision of the manuscript.

FUNDING INFORMATION

This work 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 (grant RD12/0015/0012; REIPI RD12/0015/0012), cofinanced by European Development Regional Fund "A way to achieve Europe" ERDF, and Fondo de Investigación Sanitaria (grant PI13/00063).

REFERENCES

1. **van der Starre WE, van Nieuwkoop C, Paltansing S, van't Wout JW, Groeneveld GH, Becker MJ, Koster T, Wattel-Louis GH, Delfos NM, Ablij HC, Leyten EMS, Blom JW, van Dissel JT.** 2011. Risk factors for fluoroquinolone-resistant Escherichia coli in adults with community-onset febrile urinary tract infection. J Antimicrob Chemother **66:**650–656. http://dx.doi.org/10.1093/jac/dkq465.
2. **Etienne M, Lefebvre E, Frebourg N, Hamel H, Pestel-Caron M, Caron F, Bacyst Study Group.** 2014. Antibiotic treatment of acute uncomplicated cystitis based on rapid urine test and local epidemiology: lessons from a primary care series. BMC Infect Dis **14:**137. http://dx.doi.org/10.1186/1471-2334-14-137.
3. **van den Broek d'Obrenan J, Verheij TJ, Numans ME, van der Velden AW.** 2014. Antibiotic use in Dutch primary care: relation between diagnosis, consultation and treatment. J Antimicrob Chemother **69:**1701–1707. http://dx.doi.org/10.1093/jac/dku005.
4. **Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, Moran GJ, Nicolle LE, Raz R, Schaeffer AJ, Soper DE; Infectious Diseases Society of America; European Society for Microbiology and Infectious Diseases.** 2011. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the Infectious Diseases Society of America and the European Society for Microbiology and Infectious Diseases. Clin Infect Dis **52:**e103–e120. http://dx.doi.org/10.1093/cid/ciq257.
5. **Parasuraman R, Julian K; AST Infectious Diseases Community of Practice.** 2013. Urinary tract infections in solid organ transplantation. Am J Transplant **13**(Suppl 4)**:**S327–S336. http://dx.doi.org/10.1111/ajt.12124.
6. **Aypak C, Altunsoy A, Düzgün N.** 2009. Empiric antibiotic therapy in acute uncomplicated urinary tract infections and fluoroquinolone resistance: a prospective observational study. Ann Clin Microbiol Antimicrob **8:**27. http://dx.doi.org/10.1186/1476-0711-8-27.
7. **Lautenbach E, Strom BL, Nachamkin I, Bilker WB, Marr AM, Larosa LA, Fishman NO.** 2004. Longitudinal trends in fluoroquinolone resistance among Enterobacteriaceae isolates from inpatients and outpatients, 1989–2000: differences in the emergence and epidemiology of resistance across organisms. Clin Infect Dis **38:**655–662. http://dx.doi.org/10.1086/381549.
8. **Takahashi A, Muratani T, Yasuda M, Takahashi S, Monden K, Ishikawa K, Kiyota H, Arakawa S, Matsumoto T, Shima H, Kurazono H, Yamamoto S.** 2009. Genetic profiles of fluoroquinolone-resistant Escherichia coli isolates obtained from patients with cystitis: phylogeny, virulence factors, PAIusp subtypes, and mutation patterns. J Clin Microbiol **47:**791–795. http://dx.doi.org/10.1128/JCM.01740-08.
9. **Komp Lindgren P, Karlsson A, Hughes D.** 2003. Mutation rate and evolution of fluoroquinolone resistance in Escherichia coli isolates from patients with urinary tract infections. Antimicrob Agents Chemother **47:**3222–3232. http://dx.doi.org/10.1128/AAC.47.10.3222-3232.2003.
10. **Hooper DC, Jacoby GA.** 17 July 2015. Mechanisms of drug resistance: quinolone resistance. Ann N Y Acad Sci http://dx.doi.org/10.1111/nyas.12830.
11. **Marin SJ, Coles R, Merrell M, McMillin GA.** 2008. Quantitation of benzodiazepines in urine, serum, plasma, and meconium by LC-MS-MS. J Anal Toxicol **32:**491–498. http://dx.doi.org/10.1093/jat/32.7.491.
12. **Bilobrov VM, Chugaj AV, Bessarabov VI.** 1990. Urine pH variation dynamics in healthy individuals and stone formers. Urol Int **45:**326–331. http://dx.doi.org/10.1159/000281730.
13. **Giannakopoulos X, Evangelou A, Kalfakakou V, Grammeniatis E, Papandropoulos I, Charalambopoulos K.** 1997. Human bladder urine oxygen content: implications for urinary tract diseases. Int Urol Nephrol **29:**393–401. http://dx.doi.org/10.1007/BF02551103.
14. **Völgyi G, Vizserálek G, Takács-Novák K, Avdeef A, Tam KY.** 2012. Predicting the exposure and antibacterial activity of fluoroquinolones based on physicochemical properties. Eur J Pharm Sci **47:**21–27. http://dx.doi.org/10.1016/j.ejps.2012.04.022.
15. **O'Shea R, Moser HE.** 2008. Physicochemical properties of antibacterial compounds: implications for drug discovery. J Med Chem **51:**2871–2878. http://dx.doi.org/10.1021/jm700967e.
16. **Smith SM, Eng RH, Cherubin CE.** 1988. Conditions affecting the results

of susceptibility testing for the quinolone compounds. Chemotherapy **34:** 308–314. http://dx.doi.org/10.1159/000238584.
17. **Zeiler HJ.** 1985. Influence of pH and human urine on the antibacterial activity of ciprofloxacin, norfloxacin and ofloxacin. Drugs Exp Clin Res **11:**335–338.
18. **Zhanel GG, Karlowsky JA, Davidson RJ, Hoban DJ.** 1991. Influence of human urine on the in vitro activity and postantibiotic effect of ciprofloxacin against Escherichia coli. Chemotherapy **37:**218–223. http://dx.doi.org/10.1159/000238857.
19. **Aagaard J, Gasser T, Rhodes P, Madsen PO.** 1991. MICs of ciprofloxacin and trimethoprim for Escherichia coli: influence of pH, inoculum size and various body fluids. Infection **19**(Suppl 3)**:**S167–S169.
20. **Linde H-J, Lehn N.** 2004. Mutant prevention concentration of nalidixic acid, ciprofloxacin, clinafloxacin, levofloxacin, norfloxacin, ofloxacin, sparfloxacin or trovafloxacin for Escherichia coli under different growth conditions. J Antimicrob Chemother **53:**252–257. http://dx.doi.org/10.1093/jac/dkh036.
21. **Wagenlehner FME, Kinzig-Schippers M, Sörgel F, Weidner W, Naber KG.** 2006. Concentrations in plasma, urinary excretion and bactericidal activity of levofloxacin (500 mg) versus ciprofloxacin (500 mg) in healthy volunteers receiving a single oral dose. Int J Antimicrob Agents **28:**551–559. http://dx.doi.org/10.1016/j.ijantimicag.2006.07.026.
22. **Hopkins KL, Davies RH, Threlfall EJ.** 2005. Mechanisms of quinolone resistance in Escherichia coli and Salmonella: recent developments. Int J Antimicrob Agents **25:**358–373. http://dx.doi.org/10.1016/j.ijantimicag.2005.02.006.
23. **Machuca J, Briales A, Labrador G, Díaz-de-Alba P, López-Rojas R, Docobo-Pérez F, Martínez-Martínez L, Rodríguez-Baño J, Pachón ME, Pascual Á Rodríguez-Martínez J-M.** 2014. Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in Escherichia coli. J Antimicrob Chemother **69:**3203–3215. http://dx.doi.org/10.1093/jac/dku308.
24. **Clinical and Laboratory Standards Institute.** 2015. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard— 10th ed. CLSI document M07-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
25. **So W, Crandon JL, Nicolau DP.** 2015. Effects of urine matrix and pH on the potency of delafloxacin and ciprofloxacin against urogenic Escherichia coli and Klebsiella pneumoniae. J Urol **194:**563–570. http://dx.doi.org/10.1016/j.juro.2015.01.094.
26. **Barry AL.** 1990. *In vitro* activity of the quinolones and related compounds, p 79–105. *In* Siporin C, Heifetz CL, Domagala JM (ed), The new generation of quinolones. Marcel Dekker, New York, NY.
27. **Miller LG, Mehrotra R, Tang AW.** 2007. Does in vitro fluoroquinolone resistance predict clinical failure in urinary tract infections? Int J Antimicrob Agents **29:**605–607. http://dx.doi.org/10.1016/j.ijantimicag.2006.11.021.
28. **Sánchez Navarro MD, Coloma Milano C, Zarzuelo Castañeda A, Sayalero Marinero ML, Sánchez-Navarro A.** 2002. Pharmacokinetics of ciprofloxacin as a tool to optimise dosage schedules in community patients. Clin Pharmacokinet **41:**1213–1220. http://dx.doi.org/10.2165/00003088-200241140-00005.
29. **Lewin CS, Morrissey I, Smith JT.** 1991. The mode of action of quinolones: the paradox in activity of low and high concentrations and activity in the anaerobic environment. Eur J Clin Microbiol Infect Dis **10:**240–248. http://dx.doi.org/10.1007/BF01966996.
30. **Erdogan-Yildirim Z, Burian A, Manafi M, Zeitlinger M.** 2011. Impact of pH on bacterial growth and activity of recent fluoroquinolones in pooled urine. Res Microbiol **162:**249–252. http://dx.doi.org/10.1016/j.resmic.2011.01.004.
31. **Hooper DC.** 2001. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis **7:**337–341. http://dx.doi.org/10.3201/eid0702.010239.
32. **Gocke E.** 1991. Mechanism of quinolone mutagenicity in bacteria. Mutat Res **248:**135–143. http://dx.doi.org/10.1016/0027-5107(91)90095-6.
33. **López E, Elez M, Matic I, Blázquez J.** 2007. Antibiotic-mediated recombination: ciprofloxacin stimulates SOS-independent recombination of divergent sequences in Escherichia coli. Mol Microbiol **64:**83–93. http://dx.doi.org/10.1111/j.1365-2958.2007.05642.x.
34. **Ena J, Amador C, Martinez C, Ortiz de la Tabla V.** 1995. Risk factors for acquisition of urinary tract infections caused by ciprofloxacin resistant Escherichia coli. J Urol **153:**117–120. http://dx.doi.org/10.1097/00005392-199501000-00040.
35. **Remer T, Manz F.** 1995. Potential renal acid load of foods and its influence on urine pH. J Am Diet Assoc **95:**791–797. http://dx.doi.org/10.1016/S0002-8223(95)00219-7.
36. **Remer T.** 2000. Influence of diet on acid-base balance. Semin Dial **13:**221–226.
37. **Losito A, Nunzi EG, Covarelli C, Nunzi E, Ferrara G.** 2009. Increased acid excretion in kidney stone formers with essential hypertension. Nephrol Dial Transplant **24:**137–141.
38. **Cameron MA, Maalouf NM, Adams-Huet B, Moe OW, Sakhaee K.** 2006. Urine composition in type 2 diabetes: predisposition to uric acid nephrolithiasis. J Am Soc Nephrol **17:**1422–1428. http://dx.doi.org/10.1681/ASN.2005121246.
39. **Berend K, de Vries APJ, Gans ROB.** 2014. Physiological approach to assessment of acid-base disturbances. N Engl J Med **371:**1434–1445. http://dx.doi.org/10.1056/NEJMra1003327.
40. **Maalouf NM, Sakhaee K, Parks JH, Coe FL, Adams-Huet B, Pak CY.** 2004. Association of urinary pH with body weight in nephrolithiasis. Kidney Int **65:**1422–1425. http://dx.doi.org/10.1111/j.1523-1755.2004.00522.x.
41. **Aparicio VA, Nebot E, García-del Moral R, Machado-Vílchez M, Porres JM, Sánchez C, Aranda P.** 2013. Dietas hiperproteicas y estado renal en ratas. Nutr Hosp **28:**232–237.
42. **Ferrando SJ, Levenson JL, Owen JA (ed).** 2010. Clinical manual of psychopharmacology in the medically ill. American Psychiatric Publishing, Washington, DC.
43. **Kovesdy CP.** 2012. Metabolic acidosis and kidney disease: does bicarbonate therapy slow the progression of CKD? Nephrol Dial Transplant **27:**3056–3062. http://dx.doi.org/10.1093/ndt/gfs291.
44. **Fjellstedt E, Denneberg T, Jeppsson J-O, Tiselius H-G.** 2001. A comparison of the effects of potassium citrate and sodium bicarbonate in the alkalinization of urine in homozygous cystinuria. Urol Res **29:**295–302. http://dx.doi.org/10.1007/s002400100200.
45. **Kamberi M, Tsutsumi K, Kotegawa T, Kawano K, Nakamura K, Niki Y, Nakano S.** 1999. Influences of urinary pH on ciprofloxacin pharmacokinetics in humans and antimicrobial activity in vitro versus those of sparfloxacin. Antimicrob Agents Chemother **43:**525–529.