

## Quinolone-Resistant *Escherichia coli*

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Quinolones (nalidixic acid - NAL, norfloxacin - NOR, ciprofloxacin - CIP and gatifloxacin - GAT) were tested against *Escherichia coli* isolated from urine (385 patient samples) by disk diffusion (DD) and agar dilution (AD) methods. Fifty-three samples (13.8%) were classified as resistant to at least one of the quinolones tested. CIP and NOR susceptibilities were the same (91.4%) and they were similar to GAT (92.7%). Susceptibility to NAL, detected by the disk diffusion method, was used to predict susceptibility to NOR, CIP and GAT by the agar dilution method. The sensitivity and specificity of NAL were 100% and 95%, respectively. Twelve samples were analyzed for mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes. Sequencing of these genes failed to find any mutations in the quinolone-sensitive isolates. However, three mutations were observed in the isolates resistant to all the quinolones tested - two in *gyrA* and one in *parC*. A single mutation in *gyrA* was found in the strains that were resistant to nalidixic acid but fluoroquinolone-sensitive. These findings support the suggestion that NAL could be used as a marker for susceptibility to fluoroquinolones in routine microbiology laboratories. The overall resistance rate to quinolones in the present study was 13.8%, which is higher than that observed in other studies carried out in developed countries. Our findings serve as a warning that resistance to this group of antimicrobial agents is increasing.

**Key-Words:** Urinary infection, quinolone resistance, *Escherichia coli*.

*E. coli* is the main etiologic agent of acute urinary tract infections [3,12], which are usually treated with quinolones and fluoroquinolones [2,16]. Nalidixic acid was the first clinically available quinolone. However, because of its pharmacokinetic properties and the emergence of resistant isolates, its clinical use has been abandoned [2], although it continues to be used in some Brazilian medical centers to treat urinary tract infections in the pediatric population.

The appearance of fluoroquinolone-resistant bacteria was observed immediately after the introduction of these antimicrobials into clinical practice to treat infections in hospitalized patients [16] and community-acquired infections caused by *E. coli* [1].

Resistance to fluoroquinolones occurs mainly as a result of mutations in bacterial *gyrA* and *parC* genes that code for DNA gyrase and topoisomerase IV, respectively [14-16]. Single mutations in *gyrA* or *parC* can cause resistance to the older quinolones, such as nalidixic acid, as well as a reduction in the potency of fluoroquinolones, which is reflected in an increase in the minimal inhibitory concentration (MIC) [6,8,17].

The aim of this study was to evaluate the antimicrobial activity of quinolones against *E. coli* isolated from urine as well as to establish if resistance to nalidixic acid could constitute a good marker of resistance to the newest fluoroquinolones. In addition, analyses to detect mutations

in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes were also carried out in selected isolates.

A total of 385 consecutive *Escherichia coli* isolates were obtained from the urine of patients. The patients selected exhibited urine quantitative counts ( $\geq 10^4$  CFU/mL) and were examined by physicians at the Hospital de Clínicas, Universidade Federal do Paraná (HC-UFPR), between May and November - 2002. Only one bacterial isolate per patient was included in the study. These were identified using the WalkAway® automated system (Dade Behring™, Sacramento, USA).

The antibacterial activities of nalidixic acid (NAL), norfloxacin (NOR), ciprofloxacin (CIP), and gatifloxacin (GAT) were determined by agar dilution, according to the Clinical Laboratory Standards Institute (CLSI) guidelines [4]. Nalidixic acid susceptibility testing was also performed using the disk diffusion technique as described in the CLSI guidelines [5].

Molecular characterization of quinolone resistance was carried out by amplification of *gyrA* and *parC* genes by PCR followed by sequencing of the respective amplicons [11,14] using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and ABI BioPrism 3,100 DNA analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA). Nucleotide sequences and their deduced protein product alignments were determined using the Lasergene software package (DNASTAR, Madison, WI). Most of the *E. coli* strains (91.2 %) were isolated from outpatients, and only 8.8% were isolated from hospitalized patients. The rate of susceptibility to CIP and NOR for the 385 *E. coli* isolates tested was the same (91.4%) and was slightly lower than that for gatifloxacin (no longer on the market) (92.7%) and higher than that for nalidixic acid (86.2%).

Based on the agar dilution MIC results, the 385 *E. coli* isolates were divided into three groups: group 1, 332 (86.2%)

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*E. coli* isolates susceptible to the four quinolones tested; group 2, 19 (4.9%) *E. coli* isolates resistant to nalidixic acid but susceptible to the remaining quinolones; and group 3, 34 (8.8%) *E. coli* isolates resistant to all four quinolones.

The ciprofloxacin MIC variations were evaluated among these three groups of strains according to patient gender (Table 1). An interesting finding was that *E. coli* isolates belonging to group 3 were more frequently isolated from male than female patients ( $p < 0.005$ ). In group 1, CIP MICs varied from  $\leq 0.03$   $\mu\text{g/mL}$  to  $0.12$   $\mu\text{g/mL}$ , as shown in Table 2. In this group, 329 *E. coli* isolates exhibited CIP MICs  $\leq 0.03$   $\mu\text{g/mL}$ , two strains exhibited MICs of  $0.06$   $\mu\text{g/mL}$ , and one strain exhibited a MIC of  $0.012$   $\mu\text{g/mL}$ . As expected, the 34 *E. coli* isolates classified in group 3 had MICs higher than  $2$   $\mu\text{g/mL}$ . In group 2, there was a significant increase in the CIP MICs ( $> 0.06$   $\mu\text{g/mL}$ ) compared with group 1 ( $p < 0.005$ ).

Twelve *E. coli* isolates (two randomly chosen from group 1, five randomly chosen from group 2 and five randomly chosen from group 3) were submitted to genetic characterization of the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes. The mutations found in the QRDR of the *gyrA* and *parC* genes are shown in Table 2. The two *E. coli* isolates belonging to group 1 did not show any mutations in the QRDR of the genes studied. However, the five isolates belonging to group 3 exhibited double mutations in the *gyrA* gene (codons 83 and 87) and a single mutation in the *parC* gene (codon 80). Of the five *E. coli* isolates belonging to group 2, three displayed a single mutation in the *gyrA* gene (codon 83). Curiously, one isolate from group 2 showed no mutation, while the remaining one showed a single mutation in the QRDR of the *gyrA* and *parC* genes.

A correlation was observed between NAL inhibition zones and minimal inhibitory concentrations for the fluoroquinolones (NOR, CIP, GAT), as illustrated in Figure 1. All the 333 *E. coli* isolates exhibiting NAL inhibition zones  $\geq 19$  mm were also susceptible to NOR, CIP and GAT. Among the 49 *E. coli* isolates resistant to NAL, 32, 33, and 31 isolates were categorized as resistant to NOR, CIP and GAT, respectively. The three *E. coli* isolates that exhibited intermediate resistance to NAL were susceptible to the fluoroquinolones tested.

For 99% of the *E. coli* isolates categorized as susceptible to NAL and CIP, the ciprofloxacin MICs were between  $0.015$  and  $0.03$   $\mu\text{g/mL}$ ; however, in 13 of 15 *E. coli* isolates categorized as resistant to NAL and susceptible to CIP the corresponding figure was between  $0.12$  and  $0.25$   $\mu\text{g/mL}$ . The same pattern was observed for GAT and NOR. The MICs for 94% of the *E. coli* isolates susceptible to NAL and GAT were between  $0.03$  and  $0.06$   $\mu\text{g/mL}$ , while the MICs for isolates resistant to nalidixic acid but susceptible to ciprofloxacin were above  $0.25$   $\mu\text{g/mL}$ . Thus, the *E. coli* isolates susceptible to NOR, CIP and GAT but with intermediate or full resistance to nalidixic acid showed an increase in fluoroquinolone MICs of  $\geq 2$  dilutions compared with fully susceptible *E. coli* isolates. When susceptibility to nalidixic acid was assayed by disk

diffusion to predict susceptibility to NOR, CIP and GAT, the sensitivity and specificity of nalidixic acid were 100% and 95%, respectively. The negative predictive value (NPV) was 100% for the three quinolones studied, while the positive predictive value (PPV) was 65.4% for NOR and CIP and 63.5% for GAT.

The present study has shown higher rates of susceptibility to fluoroquinolones among urinary *E. coli* isolates collected from a Brazilian medical center than previously published studies that investigated Latin American isolates [12,18]. This difference may be because most of the *E. coli* in our study were isolated from outpatients rather than hospitalized patients.

Nalidixic acid inhibition zones correlated with the MICs of NOR, CIP and GAT. This finding shows that resistance to NAL can be used as a good indicator of fluoroquinolone resistance in *E. coli*. We observed that fluoroquinolone MICs for isolates that were resistant to NAL but susceptible to other fluoroquinolones were higher, indicating decreased susceptibility to these drugs. In addition, the level of resistance to quinolones depends on the number of accumulated mutations in the QRDR of the *gyrA* and *parC* genes [20]. Other resistance mechanisms, such as efflux pumps and alteration in outer membrane permeability, may also be involved.

A further interesting observation is that the number of nalidixic acid-resistant strains susceptible to fluoroquinolones was higher among female patients, while the number of *E. coli* isolates that were fully resistant to quinolones was higher in men, perhaps because of the association between urinary tract infections and prostatitis, where quinolones are widely used.

Bacteria with reduced susceptibility to quinolones commonly show mutations in the *gyrA* gene, which encodes the subunit *gyrA* of the DNA gyrase enzyme. These mutations occur in a region known as the "quinolone resistance-determining region" (QRDR), which is located between nucleotides 201 and 320 and encodes amino acid residues 67 to 106 in *E. coli gyrA*. This is the main mechanism of quinolone-resistance in *Enterobacteriaceae*. Mutations in the *gyrB* gene have been reported, although they appear to play a minor role in conferring resistance to quinolones [10,15,16]. Mutations in the genes that encode topoisomerase IV, particularly the *parC* gene, correlate with reduced susceptibility to quinolones. Generally these mutations occur in amino acid positions 78, 80 and 84, with the substitution of glycine by aspartate, serine by arginine or isoleucine and glycine by aspartate, respectively. In the *parE* gene, mutations leading to quinolone resistance are not common, although the substitution of leucine by histidine has been detected at position 445 [10,17,20].

In order to analyze the correlation between genetic characterization and resistance phenotype, ten quinolone-resistant and two quinolone-susceptible *E. coli* strains were submitted to amplification and sequencing of the *gyrA* and *parC* QRDR genes (Table 2). The isolates were divided into groups (1, 2 and 3) according to the quinolone susceptibility

**Table 1.** Distribution of ciprofloxacin minimal inhibiting concentrations in 385 *E. coli* isolates

Gender	Group	Ciprofloxacin (µg/mL)												N		
		0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32		64	128
Female	1	218	72													290
	2		2	3	9	3										17
	3								1	3	2	7	3	4		20
																327
Male	1	30	9	2	1											42
	2				2											2
	3							1		4	6	1	2			14
																58
<b>Total</b>																385

Group 1 - *E. coli* isolates susceptible to the four quinolones tested; group 2 - isolates resistant to nalidixic acid but susceptible to the other quinolones; and group 3 - isolates resistant to all four quinolones. N - number of isolates.

**Table 2.** Location and type of mutations in *gyrA* and *parC* genes found in selected *E. coli* isolates

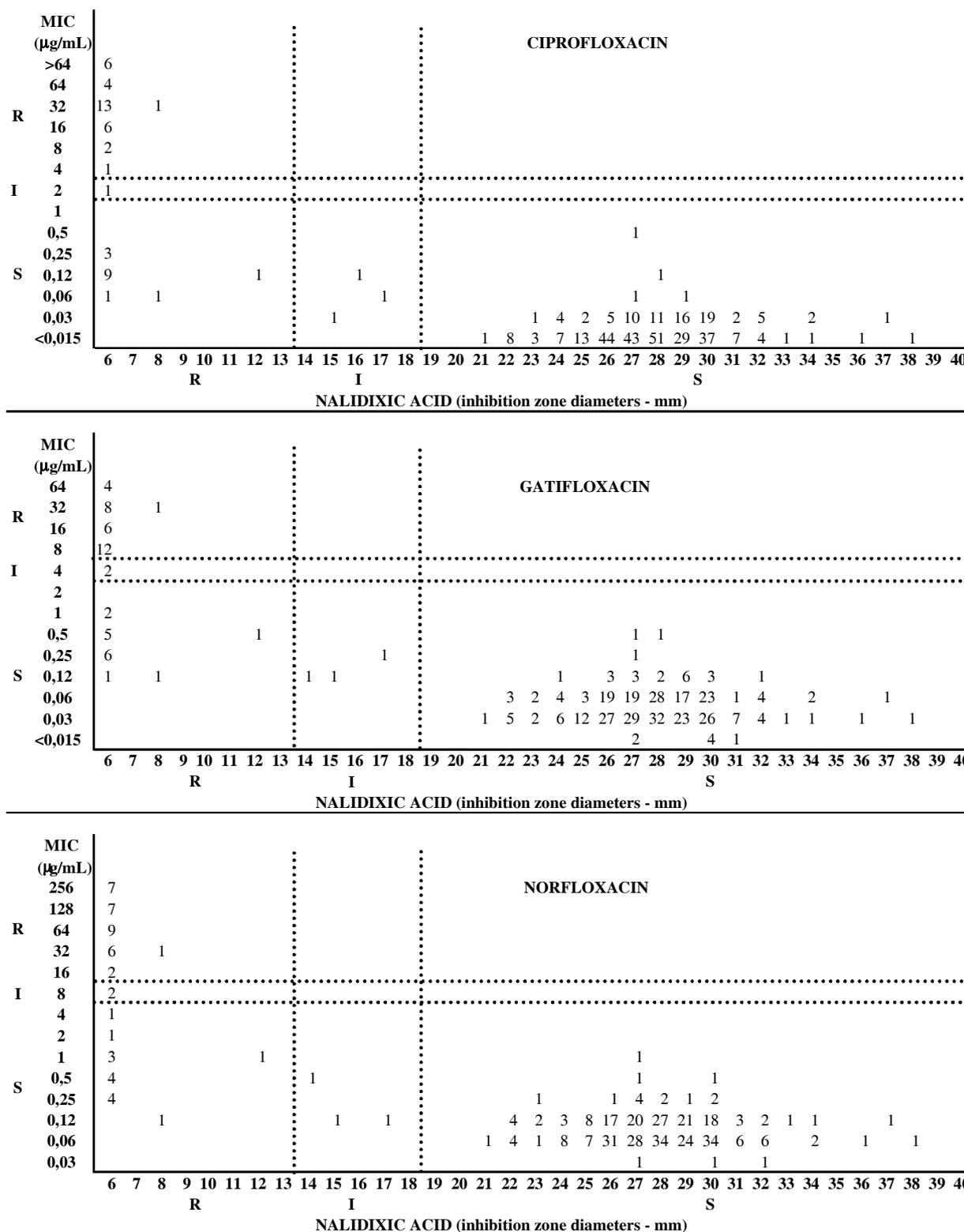
Strain	Group	MIC (µg/mL)		Mutation						
		NAL	CIP	<i>gyrA</i>			<i>parC</i>			
				83	87	80	Asp	Ser	Ala	
5384	1	1	0.03	..-.....-.....-.....-.....-..				..-.....-.....-..		
9850	1	1	0.015	..-.....-.....-.....-.....-..				..-.....-.....-..		
837	2	64	0.03	..-.....-.....-.....-.....-..				..-.....-.....-..		
4633	2	64	0.25	Leu.....-.....-.....-.....-..				..-.....-.....-..		
9233	2	64	0.12	Leu.....-.....-.....-.....-..				..-.....-.....-..		
4557	2	>512	0.12	Leu.....-.....-.....-.....-..				..-.....-.....-..		
8889	2	>512	0.25	Leu.....-.....-.....-.....-..				..-.....Arg.....-..		
6556	3	>512	8	Leu.....-.....-.....-.....Asn				..-.....Ile.....-		
345	3	>512	64	Leu.....-.....-.....-.....Asn				..-.....Ile.....-		
6723	3	>512	64	Leu.....-.....-.....-.....Asn				..-.....Ile.....-		
5850	3	>512	4	Leu.....-.....-.....-.....Asn				..-.....Ile.....-		
6452	3	>512	32	Leu.....-.....-.....-.....Asn				..-.....Ile.....-		

Group 1 - *E. coli* isolates susceptible to the four quinolones tested; group 2 - isolates resistant to nalidixic acid but susceptible to the other quinolones; and group 3 - isolates resistant to all four quinolones. NAL - nalidixic acid; CIP - ciprofloxacin.

phenotype; the MICs for ciprofloxacin of the isolates varied. Of the five strains belonging to Group 2, three *E. coli* isolates showed a single mutation in the *gyrA* gene at position 83 (Ser→Leu) and one *E. coli* strain showed two mutations, one in *gyrA* at position 83 (Ser→Leu) and another at position 80 of the *parC* gene (Ser→Arg). *E. coli* isolates with a single mutation in *gyrA* had increased fluoroquinolone MICs, but these were not high enough to indicate resistance according to the CLSI breakpoints. However, these isolates were fully resistant to nalidixic acid. These results are in agreement with those previously reported [9,11], indicating that nalidixic acid could be a good marker for single mutations, even when testing is carried out by the disk diffusion method.

The *E. coli* strains belonging to group 3 had three mutations: two in the *gyrA* gene and one in the *parC* gene. This confirms that multiple mutations are necessary to express high levels of resistance to quinolones. The five strains resistant to CIP encoded three mutations, two in *gyrA* at positions 83 (Ser→Leu) and 87 (Asp→Asn) and one in *parC* at position 80 (Ser→Ile). Only one *E. coli* isolate was resistant to nalidixic acid but susceptible to ciprofloxacin (MIC of 0.25 µg/mL). It displayed two mutations, one in position 83 (Ser→Leu) of *gyrA* and the second at position 80 (Ser→Arg) of *parC*. This result, which was also observed by other authors, supports the supposition that it is necessary to accumulate mutations in the primary resistance gene to confer a high level of resistance to quinolones [7,15,16].

**Figure 1.** Correlation between inhibition zone of nalidixic acid (mm) and MICs ( $\mu\text{g/mL}$ ) of norfloxacin, ciprofloxacin and gatifloxacin



The overall resistance rate to quinolones in the present study was 13.8%. Although this rate is lower than those reported by other Latin American studies, it is higher than those observed in other studies performed in developed countries [12,19]. Our findings serve as a warning that resistance to these groups of antimicrobial agents is increasing.

Testing for susceptibility to nalidixic acid was shown to be useful as a good predictive marker of susceptibility to fluoroquinolones in *E. coli*. The sensitivity of this screening method was as high as 100% with a specificity of 95%. These results support the suggestion that nalidixic acid could be used as a marker for susceptibility to fluoroquinolones in routine microbiology laboratories.

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