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MUTATIONS IN THE QUINOLONE RESISTANCE-DETERMINING REGIONS OF *GYRA* AND *PARC* IN *ENTEROBACTERIACEAE* ISOLATES FROM BRAZIL

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ABSTRACT

Mutations in the quinolone resistance-determining regions (QRDR) in chromosomal g*yrA* and *parC* genes and fluoroquinolone susceptibility profiles were investigated in quinolone-resistant *Enterobacteriaceae* isolated from community and hospitalized patients in the Brazilian Southeast region. A total of 112 nalidixic acid-resistant enterobacterial isolates collected from 2000 to 2005 were investigated for mutations in the topoisomerases genes *gyrA* and *parC* by amplifying and sequencing the QRDR regions. Susceptibility to fluoroquinolones was tested by the agar dilution method. Amongst the 112 enterobacterial isolates, 81 (72.3%) were resistant to ciprofloxacin and 5 (4.5%) showed reduced susceptibility. Twenty-six (23.2%) were susceptible to ciprofloxacin. Several alterations were detected in *gyrA* and *parC* genes. *Escherichia coli* isolates (47.7%) showed double mutations in the *gyrA* gene and a single one in the *parC* gene. Two unusual aminoacid substitutions are reported, an Asp87-Asn in a *Citrobacter freundii* isolate with reduced susceptibility to fluoroquinolones and a Glu84-Ala in one *E. coli* isolate. Only a *parC* gene mutation was found in fluoroquinolone-susceptible *Enterobacter aerogenes*. None of the isolates susceptible to ciprofloxacin presented mutations in topoisomerase genes. This comprehensive analysis of QRDRs in *gyrA* and *parC* genes, covering commonly isolated *Enterobacteriaceae* in Brazil is the largest reported up to now.

Key words: quinolone resistance-determining region, mutations, *gyrA*, *parC*, *Enterobacteriaceae*.

INTRODUCTION

Fluoroquinolones have been frequently prescribed as empirical therapy against most hospital and community infections due to increased appearance of multiple drug resistant Gram-negative pathogens and to the disease severity (2, 8, 22). In countries with extensive clinical use of quinolones, fluoroquinolone resistance has been a problem in clinical medicine for its limiting of available agents in the treatment of many types of infection (1, 7, 15, 23).

Brazil has one of the highest rates of resistance to quinolones among Latin American countries. A report from the SENTRY Antimicrobial Surveillance Program revealed that resistance to nalidixic acid was higher in Latin American

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(15%) than in North America (6.3%), being as high as 50% in Mexico and 33.6% in Brazil (1). Brazilian investigators confirmed the information reporting that the significant increase in resistance to quinolones detected in Brazilian medical centers between 2002-2006 may be related to increased drug use in community acquired infections and hospital-wide antibiotic pressure (9, 18).

Fluoroquinolone resistance has been associated with DNA gyrase and topoisomerase IV alterations for both Gram-negative and Gram-positive organisms. Structural topoisomerase changes reducing the affinity of this enzyme to fluoroquinolones are caused by mutations in the quinolone resistance-determining regions (QRDR) of *gyrA* and *parC* genes (14, 21). Other mechanisms involve mutations affecting the intracellular accumulation of fluoroquinolones in the cell wall (19) and the expression of outer membrane proteins (OMP) leading to reduced entry of antibiotics (17, 24). Three additional mechanisms of quinolone resistance have been reported. These refer to the presence of Qnr proteins, which protect type II topoisomerases from quinolones (10, 11, 16); of a variant aminoglycoside acetyltransferase, Aac(6')-Ib-cr, that modifies ciprofloxacin (20) and of active drug efflux pumps QepA and OqxAB, belonging to the major facilitator superfamily (MFS) transporters (6, 27). Topoisomerase mutations have not been, up to now, systematically investigated and explored in clinical isolates from Brazil, except in a report on ciprofloxacin susceptible *E. coli* (5).

The objective of this study was to assess the presence of mutations in the QRDR of chromosomal g*yrA* and *parC* genes and fluoroquinolone susceptibility profile in quinolone-resistant E*nterobacteriaceae* isolated from community and hospital patients in the Brazilian Southeast region.

MATERIALS AND METHODS

Bacterial isolates and susceptibility testing

From 2000 to 2005, 112 clinical non-duplicate nalidixic acid- resistant *Enterobacteriaceae* isolates were obtained from outpatients (n=91) and inpatients (n=21) referred to laboratories located in the city of Juiz de Fora (State of Minas Gerais) and Ribeirão Preto (State of São Paulo). All species were identified both by conventional techniques and by MiniAPI, a semi-automatized assay (bioMérieux, Marcy l'Etoile, France). Susceptibility to fluoroquinolones was tested by the agar dilution method with *E. coli* ATCC 25922 as a control reference in MIC determinations. Susceptibility and resistance breakpoints were those defined by the Clinical and Laboratory Standards Institute (3).

Detection of mutations in the topoisomerase genes

DNA extraction and PCR analysis were performed as described elsewhere using sets of primers designed for amplification and sequencing of topoisomerase genes (10). DNA was amplified using an initial denaturation step of 5 min at 94^{o}C, followed by 30 cycles consisting of 1 min at 94^{o}C, 1 min at the annealing temperature of 55^{o}C and 1 min at 72^{o}C, and a final extension step of 10 min at 72^{o}C. Amplified DNA products were resolved by electrophoresis in 1% agarose gels and staining with ethidium bromide. PCR products were purified for sequencing by GFX^{TM} PCR (GE Healthcare, Buckinghamshire, UK). The forward and reverse strands were sequenced in MegaBACE 1000 (GE Healthcare) with the same PCR primer sets. Raw sequences were reviewed by visual inspection using the software Chromas v.1.45 (32-bit) (Technelysium Pty. Ltd). QRDR nucleotide sequences in *gyrA* and *parC* genes from each of the 112 isolates were compared with the respective reference sequences in the GenBank database (accession numbers from AF052253 to AF052260).

RESULTS AND DISCUSSION

Species distribution among the 112 *Enterobacteriaceae* isolates was: *Escherichia coli* (n=65), *Klebsiella pneumoniae* (n=21), *Klebsiella oxytoca* (n=12), *Enterobacter cloacae* (n=6), *Citrobacter freundii* (n=2), *Providencia stuartii* (n=2), *Serratia marcescens* (n=2), *Enterobacter aerogenes* (n=1) and *Proteus*

mirabilis (n=1). Twenty-three percent of the 112 nalidixic acid-resistant isolates were susceptible to ciprofloxacin and/or levofloxacin (MIC ≤ 1 µg/mL).

Table 1 summarizes codon numbers and the corresponding alteration detected in the *gyrA* and *parC* genes of fluoroquinolone-resistant and susceptible clinical isolates included in the study and the corresponding MIC values. All *Enterobacteriaceae* isolates had amplification products.

Table 1. Fluoroquinolone MIC values, and alterations detected in the *gyrA* and *parC* genes of *Enterobacteriaceae* isolates

Species	Number of isolates	MIC (µg/mL) †‡				*gyrA* position			*parC* position	
		CIP	NOR	OFL	LVX					
Citrobacter freundii	1	32	64	64	32	Thr83 Ile	Asp87Asn			
	1	0.25	0.5	1	0.5	Thr83 Ile				
Enterobacter aerogenes	1	0.5	1	0.5	0.25					Cys107Trp
Enterobacter cloacae	1	16	64	16	8	Ser83Phe	Asp87Asn	Ser80Ile		
	2	32-64	64-128	16-64	8-64	Ser83Phe	Asp87Asn			
	1	64	>128	32	32	Ser83Tyr	Asp87Ala			
	1	0,125	1	2	0,5	---------- No mutation ----------				
	1*	2	1	1	0,5	---------- No mutation ----------				
Escherichia coli	4	32 - >128	>128	64-128	16-128	Ser83Leu	Asp87Asn	Ser80Ile	Glu84Gly	
	1	32	32	>128	16	Ser83Leu	Asp87Asn	Ser80Ile	Glu84Ala	
	1	16	128	64	32	Ser83Leu	Asp87Asn	Ser80Ile	Glu84Lys	
	1	64	128	128	64	Ser83Leu	Asp87Asn	Ser80Arg		
	31	2->128	8 - >128	4-128	2-64	Ser83Leu	Asp87Asn	Ser80Ile		
	4	2	4	1-4	1-2	Ser83Leu	Asp87Tyr	Ser80Ile		
	1	1	4	2	1	Ser83Leu	Asp87Gly	Gly78Cys		
	1	8	16	16	8	Ser83Leu	Asp87Gly	Ser80Ile		
	7	8-64	16-128	16-64	4-64	Ser83Leu	Asp87Asn		Glu84Lys	
	1	1	2	4	2	Ser83Leu	Asp87Asn			Cys107Trp
	1	4	8	8	4	Ser83Leu		Ser80Arg		
	3	0.06-1	0.5-4	0.12-4	0.12- 2		Asp87Gly			
	4	0.03-8	0.5-64	0.5-8	0.25-8	Ser83Leu				
	5	0.03-1	0.03-4	0.06-2	0.03-4	---------- No mutation ----------				
Klbsiella oxytoca	1	32	64	64	32	Thr83Ile	Asp87Tyr	Ser80Arg	Pro91Ser	
	6	16-32	64-128	16-32	8-16	Thr83Ile	Asp87His	Ser80Ile	Pro91Ser	
	1	32	64	64	32	Thr83Ile	Asp87Tyr		Pro91Ser	
	4	8-16	16-64	8-16	2-8	Thr83Ile	Asp87His	Ser80Ile		
Klebsiella pneumoniae	1	16	64	32	16	Ser83Phe	Asp87Ala	Ser80Ile		
	4	4-8	16-64	4-16	4-8	Ser83Ile		Ser80Ile		
	1	128	>128	128	128	Ser83Phe		Ser80Ile		
	8	0.25-4	1-16	1-8	0.5-4	Ser83Phe				
	3	4-8	16-32	4-16	2-8	Ser83Tyr				
	1	1	8	4	2		Asp87Tyr			
	3	0.5-1	0.5-8	0.125-4	0.06-1	---------- No mutation ----------				
Proteus mirabilis	1	1	8	1	1	Ser83Phe				
Providencia stuartii	1	64	128	64	128	Ser83Ile	Glu87Gly			
	1	32	>128	16	16	Ser83Arg				
Serratia marcescens	2	8-16	64-128	16-32	16	Ser83Ile				

* *qnrA* gene present

† CIP, ciprofloxacin, NOR, norfloxacin; OFL, ofloxacin; LVX, levofloxacin

‡ Susceptibility (≤ value) and resistance (≥ value) breakpoints defined by CLSI (2011): 4 µg/mL and 16 µg/mL for norfloxacin, 2 µg/mL and 8 µg/mL for levofloxacin and ofloxacin, 1 µg/mL and 4 µg/mL for ciprofloxacin.

According to Ruiz (21), codons 83 and 87 in *gyrA* and codons 80 and 84 in p*arC* gene display the most common alterations in clinical isolates. In addition, expression of high-level fluoroquinolone resistance in *Enterobacteriaceae* requires the presence of multiple mutations in *gyrA* and/or *parC* genes.

Topoisomerase genes in nine isolates susceptible to

ciprofloxacin (MICs ≤ 1 μg/mL) did not show mutations. However, one *E. cloacae* exhibiting decreased susceptibility to fluoroquinolones (MIC 2 μg/mL) and no mutation in topoisomerase genes had a previously detected *qnrA* gene (4, 12).

The majority of *Enterobacteriaceae* isolates in the study (91%) showed a QRDR region with mutations in codons 83 and/or 87 of the *gyrA* gene. Twelve different types of mutations were found amongst the collection analyzed. They were Thr83-Ile and Asp87-Asn in *C. freundii*, Ser83-Phe, Ser83-Tyr, Asp87-Asn and Asp87-Ala in *E. cloacae*, Ser83-Leu, Asp87-Asn, Asp87-Tyr and Asp87-Gly in *E. coli*, Thr83-Ile, Asp87-Tyr and Asp87-His in *K. oxytoca*, Ser83-Phe, Ser83-Tyr, Asp87-Ala and Asp87-Tyr in *K. pneumoniae*, Ser83-Phe in *P. mirabilis*, Ser83-Ile, Ser83-Arg and Glu87-Gly in *P. stuartii* and Ser83-Ile in *S. marcescens* (Table 1). All fluoroquinolone resistant *E. coli* isolates had the usual Ser83-Leu substitution in *gyrA* gene but according to Vila *et al.* (25), the additional mutation in codon 87 is associated with an increased fluoroquinolone resistance. This was confirmed by the results for *E. coli* (Table 1), as four of the isolates with a single alteration, Ser83-Leu, had MICs ≤ 8 μg/mL for ciprofloxacin. However, the isolates with an additional and common mutation in codon 87(Asp87-Asn), as shown by 91.6% of the samples, had higher ciprofloxacin MICs ≥ 8 μg/mL. Eleven *K. pneumoniae* isolates with a single alteration in codon 83 also showed ciprofloxacin MICs < 8 μg/mL. Three *E. coli* isolates and one *K. pneumoniae* having a single alteration in codon 87, Asp87-Gly or Asp87-Tyr, had MIC ≤ 1 μg/mL for ciprofloxacin.

Codons 78, 80, 84 and 107 showed alterations in the QRDR of the *parC* gene. The replacements were Gly78-Cys, Ser80-Ile, Ser80-Arg, Glu84-Gly, Glu84-Ala, Glu84-Lys, Cys107-Trp in *E. coli*, Ser80-Ile in *E. cloacae*, Cys107-Trp in *E. aerogenes*, Ser80-Arg, Ser80-Ile, Pro91-Ser, in *K. oxytoca* and Ser80-Ile in *K. pneumoniae*. Analysis of the data presented in Table 1, did not indicate that these changes could be by themselves responsible for the difference in fluoroquinolone MIC values among the different isolates.

A different substitution, not reported to date, at codon 84 of the *parC* gene (glutamine replaced by alanine) was exhibited by one *E. coli* isolate with Ser83-Leu, Asp87-Asn and Ser80-Ile replacements. *C. freundii*, *P. mirabilis*, *P. stuartii, S. marcescens* isolates did not present mutations in *parC* gene.

Substitutions in *gyrA* and *parC* genes were found in 95.8% of fluoroquinolones-resistant *E. coli* isolates in this study. According to Table 1, 47.7% of *E. coli* isolates showed substitutions: Ser83-Leu and Asp87-Asn in *gyrA* gene and Ser80-Ile in p*arC* gene, while six isolates (9.2%) (CIP MIC ≥16 μg/mL) had Ser83-Leu and Asp87-Asn substitutions in g*yrA* gene, and Ser80-Ile and Glu84-Gly, Glu84-Ala or Glu84-Lys in p*arC* gene. Another uncommon substitution (serine by arginine) at codon 80 in *parC* gene was observed in one *E. coli* isolate. In general, a substitution in Ser-83 is sufficient to generate a high level of resistance to nalidixic acid, whereas a second mutation at Asp-87 in *gyrA* gene may play a complementary role in the development of high levels of ciprofloxacin resistance.

According to Weigel *et al.* (26), *C. freundii* isolates with reduced susceptibility to fluoroquinolones exhibited Thr83 to Ile mutations as a result of C-to-T substitutions at the nucleotide 248th position in *gyrA*. In one *C. freundii* isolate, in this study, another different substituted amino acid was detected in g*yrA* gene, Asp87-Asn instead of glycine, which to our knowledge has not been described so far. Although rarely reported in the literature, alterations in codon 83 (serine for isoleucine) and 87 (asparagine for alanine or tyrosine) in g*yrA* gene were detected in *K. pneumoniae* isolates (4, 26). No such alteration in the *parC* gene of the ciprofloxacin susceptible *K. pneumoniae* was found.

Just one alteration, Thr83-Ile, in *gyrA* gene was reported by Weigel *et al.* (26) in ciprofloxacin resistant *K. oxytoca*. Additional substitutions were found in this study, Asp87-Tyr in 2 isolates and Asp87-His in 10 others having MICs for ciprofloxacin of 32 μg/mL and 8 -16 μg/mL respectively. All fluoroquinolone resistant *K. oxytoca* isolates had mutations

detected in *gyrA* and *parC* genes, the most frequent being Ser80-Ile and Pro91-Ser. The alterations found in *P. stuartii* and *S. marcescens* isolates were the same reported by Weigel *et al*. (26).

It has been shown that the primary quinolone target in *Enterobacteriaceae* is gyrase and topoisomerase IV enzymes, since mutations in *parC* or *parE* are observed only in addition to the ones in DNA gyrase (7). However, the only fluoroquinolone susceptible *E. aerogenes* tested had just one mutation in codon 107 in *parC* gene, Cys107-Trp.

A diversity of alterations has been found in *gyrA* and p*arC* genes, but there was not a clear correlation between frequency of distribution of each alteration type and fluoroquinolone MIC values, which varied from 2 to >128 µg/mL for ciprofloxacin, from 4 to 128 µg/mL for ofloxacin and from 2 to 64 µg/mL for levofloxacin.

Consistent with previous studies, low-level fluoroquinolone resistance in *E. coli* is associated with a single alteration in the *GyrA* protein while high-level resistance required double mutations (2, 23). However, in all other species in this study, there were isolates exhibiting a single alteration in *gyrA* gene and high-level fluoroquinolone resistance. Other mechanisms implicated in fluoroquinolone resistance, which might be responsible for the differences in MICs values for fluoroquinolones observed among the isolates with the same types of mutation in all genes were not considered in the present study. Changes in permeability and active efflux are mechanisms that cannot be excluded and may enhance development of resistance and contribute to the selection of fluoroquinolone-resistant isolates in the course of treatments with these antibiotics. Furthermore, the presence of one or more related clones in the collection studied was not determined by molecular typing methodologies.

In conclusion, this study documented alterations in the QRDR segments of g*yrA* and p*arC* genes in ciprofloxacin susceptible and resistant *Enterobacteriaceae* recovered from clinical specimens in the Southeast region of Brazil. The alterations reported are the most detailed in the QRDR in *gyrA* and *parC* genes and include the ones in commonly isolated *Enterobacteriaceae*. Additional data on topoisomerase sequences and mutations associated with fluoroquinolone resistance in clinical isolates of diversified *Enterobacteriaceae* species was also provided.

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