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In vitro selection of resistance in *Streptococcus pneumoniae* at *in vivo* fluoroquinolone concentrations

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***Objectives*: To compare the ability to select for resistance in *Streptococcus pneumoniae* of levofloxacin, moxifloxacin, ciprofloxacin and prulifloxacin.**

***Methods*: Twenty strains of *S. pneumoniae* susceptible to fluoroquinolones were used. The frequencies of spontaneous single-step mutations at plasma and epithelial lining fluid (ELF) peak and trough antibiotic concentrations were calculated. Multi-step selection of resistance was evaluated by performing 10 serial subcultures on agar plates containing a linear gradient from peak to trough antimicrobial concentrations, followed by 10 subcultures on antibiotic-free agar. Resistant strains selected after multi-step selection were characterized for DNA mutations by sequencing *gyrA*, *gyrB*, *parC* and *parE* genes.**

***Results*: Levofloxacin and moxifloxacin showed the lowest frequencies of mutations (median $<10^{-11}$) at plasma peak and at ELF concentrations, while medians ranging from 10^{-8} to 10^{-6} were observed for ciprofloxacin and prulifloxacin. In a multi-step selection assay, ciprofloxacin and prulifloxacin selected for the highest number of resistant strains (19 and 31, respectively). No selection of resistance was observed for levofloxacin at ELF concentrations and for moxifloxacin at plasma and ELF concentrations. Mutations in *parC*, *parE* and *gyrA* genes were found in ciprofloxacin- and prulifloxacin-resistant strains, while only *parC* mutations were found for levofloxacin.**

***Conclusions*: Levofloxacin and moxifloxacin are characterized by a lower propensity to select *in vitro* for resistance in *S. pneumoniae* than ciprofloxacin and prulifloxacin, when tested at plasma and lung concentrations.**

Keywords: levofloxacin, ciprofloxacin, moxifloxacin, prulifloxacin, frequency of mutation

Introduction

Streptococcus pneumoniae is a leading bacterial cause of community-acquired pneumonia (CAP), acute sinusitis, otitis media and meningitis; however, today pneumococci are frequently resistant to many of the commonly prescribed antibiotics used to treat these infections.[1–3] The CDC estimated that 34% of the 37000 cases of invasive pneumococcal disease in 2002 were due to *S. pneumoniae* that was resistant to at least one drug, while 17% of infections were caused by pneumococci that was resistant to three or more drugs.[4]

The rapid spread of pneumococcal clones resistant to β-lactams and macrolides has led some authors to suggest that the use of fluoroquinolones might be appropriate for the treatment of pneumococcal infections.[2,5] However, an increment in fluoroquinolone-resistant *S. pneumoniae* associated with an increase in fluoroquinolone usage, ciprofloxacin being the predominant fluoroquinolone used during the study period, was already reported in 1999.[6] Despite concerns about a rapid increase in resistance to these antibiotics,[7,8] US and Italian surveillance data have shown, year after year, that resistance to the respiratory fluoroquinolones (i.e. levofloxacin and moxifloxacin) in *S. pneumoniae* is ∼1% to 3% or less, with minimal or no yearly increase.[5] Nonetheless, failures of fluoroquinolone therapy have been concurrent with the emergence of highly resistant pneumococcal strains following suboptimal initial therapy.[8]

Fluoroquinolone resistance occurs in a stepwise fashion with mutations being observed first in the quinolone resistance-determining region (QRDR) of either *parC/E* or *gyrA/B*, depending on the pathogen, the selecting drug and its concentration at

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the site of infection.[9] Mutations up-regulating active efflux have been also involved in clinical isolates, particularly in determining resistance to ciprofloxacin.[10]

In vitro studies evaluating the abilities of different fluoroquinolones to select for resistant strains of *S. pneumoniae* were not conclusive as to which respiratory fluoroquinolone has the lowest potential to select for resistant mutants.[11,12] Moreover, the majority of these studies have assessed antimicrobial concentrations that were quite different from those actually attained at the site of infection. For these reasons, we have recently modified methodologies used *in vitro* to assess selection for resistance by testing antimicrobial maximal concentrations reported to occur *in vivo*.[13] The aim of the present study was to compare the ability of levofloxacin, moxifloxacin, ciprofloxacin and prulifloxacin, an antibiotic recently launched into the Italian market, to select *in vitro* for resistance in *S. pneumoniae* clinical isolates at concentrations occurring in plasma and lung.

Materials and methods

Strains

Twenty clinical isolates of *S. pneumoniae* collected from outpatients with community-acquired respiratory tract infections in 2005 were included in the study. Susceptibility to the drugs under evaluation was considered as a prerequisite for the study. One isolate per patient was used in order to avoid inclusion of the same strain. All isolates were stored at −80°C in brain heart infusion (BHI) broth containing 10% (w/v) glycerol until use.

Antibiotics

Levofloxacin (Sanofi-Aventis, S.p.A., Milan, Italy), moxifloxacin, ciprofloxacin (Bayer Italia, S.p.A., Milan, Italy) and prulifloxacin (Aziende Chimiche Riunite Angelini Francesco ACRAF S.p.A, S. Palomba-Pomezia, Italy) were used to prepare stock solutions at concentrations of 5120 mg/L. Ciprofloxacin, levofloxacin and moxifloxacin were prepared in accordance with CLSI standards,[14] while prulifloxacin was dissolved in dimethylsulphoxide. Plasma and epithelial lining fluid (ELF) maximum and minimum concentrations (C_{max} and C_{min}) of each antimicrobial studied were chosen from those obtained in previously published studies after oral administration (Table 1).[15–18] For levofloxacin, concentrations obtained with 500 and 750 mg doses were used, since the latter has been recently introduced in the American guidelines for the treatment of CAP.[19]

Table 1. Antibiotic concentrations used throughout the study

	Plasma		ELF	
Drug	C_{max} (mg/L)	C_{min} (mg/L)	C_{max} (mg/L)	C_{min} (mg/L)
CIP	2.11[15]	0.08[15]	1.87[15]	0.41[15]
LVX 500 mg	5.29[15]	0.6[15]	9.94[15]	0.7[15]
LVX 750 mg	11.98[15]	1.69[15]	22.72[15]	1.45[15]
MXF	3.23[16]	0.78[16]	10.52[16]	5.71[16]
PRU	2[17]	0.1[18]	1.24[17]	0.48[18]

ELF, epithelial lining fluid; C_{max}, peak antibiotic concentration; C_{min}, minimum antibiotic concentration; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; PRU, prulifloxacin.

Determination of MIC

Antibiotic susceptibilities to the study drugs were determined by the microdilution broth assay in accordance with the CLSI approved standards.[14] Resistance to levofloxacin and moxifloxacin was defined as an MIC of ≥8 and ≥4 mg/L, respectively.[14] Since no CLSI breakpoints for ciprofloxacin and prulifloxacin against *S. pneumoniae* were available, reduced susceptibility to ciprofloxacin and prulifloxacin was defined as an MIC of ≥4 mg/L.[20,21]

Single-step selection of resistance

Colonies from an overnight culture on 5% sheep blood agar plates were resuspended in BHI broth at a load of ~10^{10} cfu/mL. An aliquot of 100 μL from bacterial suspension was seeded onto 5% sheep blood agar plates containing antibiotics at plasma and ELF C_{max} and C_{min}, as reported above. After 72 h of incubation in 10% CO_2-enriched atmosphere, the frequency of mutation was calculated from the ratio between colonies grown on antibiotic-containing plates and the initial inoculum, determined by plating 100 μL of bacterial suspension, after proper dilution, onto 5% sheep blood agar plates. Five colonies from each antibiotic-containing plate were randomly selected and their MIC of the corresponding antibiotic was determined as described above.

Multi-step selection of resistant bacteria

The ability to select for antibiotic resistance was evaluated by performing serial subcultures on 5% sheep blood agar plates, containing a gradient ranging from C_{max} to C_{min}. Gradients were prepared in Petri dishes, on which two layers of agar were poured. The bottom layer consisted of sheep blood agar containing the antibiotic at C_{min} allowed to harden with the plate slanted sufficiently to cover the entire bottom. The top layer, added to the dish in the normal position, contained antibiotics at C_{max}.

An inoculum of 10^{10} cfu/mL of each strain was homogenously spread onto each plate and incubated for 48 h at 37°C in a CO_2-enriched atmosphere. After incubation, colonies growing at the highest drug concentration were sampled, checked for purity, plated on antimicrobial-free agar plates and re-plated on a new agar plate containing an antibiotic gradient, prepared as described above. A total of 10 consecutive passages on antibiotic-containing plates were followed by 10 passages on antibiotic-free plates by streaking single colonies in order to evaluate the stability of acquired resistance. MIC values were determined after 1, 5 and 10 passages on antibiotic-containing plates, and after 5 and 10 passages in antibiotic-free medium. Acquisition of resistance was defined as an MIC value higher than the resistance breakpoint.

Characterization of acquired resistance

To determine whether mutants that had acquired stable resistance to quinolones had alterations in topoisomerase IV or DNA gyrase, *parC*, *parE*, *gyrA* and *gyrB* were amplified by PCR and sequenced as described previously.[22]

Amplification products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Milan, Italy) following the manufacturer's instructions. Sequencing was performed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Monza, Italy).

Only mutations known to be associated with resistance to fluoroquinolones in *S. pneumoniae* were considered (Ser81, Glu85 and Ser114 in the *gyrA* gene, Gly406 and Pro454 in the *gyrB* gene, Ser79, Asp83, Asn91 and Ala115 in the *parC* gene and Arg447, Ile460 and Ile493 in the *parE* gene).[23,24]

Table 2. Frequency of mutation at plasma and ELF antimicrobial concentrations in *S. pneumoniae* ($n=20$)

Drug	Frequency of mutation			
	C_{max} plasma	C_{min} plasma	C_{max} ELF	C_{min} ELF
LVX 500 mg				
range	$<10^{-11}$–1.92×10^{-9}	8.93×10^{-7}–9.8×10^{-6}	$<10^{-11}$	$<10^{-11}$–1.57×10^{-7}
median	$<10^{-11}$	2.09×10^{-7}	$<10^{-11}$	2.74×10^{-7}
LVX 750 mg				
range	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$
median	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$
MXF 400 mg				
range	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$
median	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$	$<10^{-11}$
CIP 500 mg				
range	$<10^{-11}$–3.45×10^{-6}	1.88×10^{-6}–7.98×10^{-5}	$<10^{-11}$–6.0×10^{-5}	7.24×10^{-9}–4.2×10^{-5}
median	1.11×10^{-7}	3.48×10^{-6}	1.68×10^{-6}	3.59×10^{-6}
PRU 600 mg				
range	$<10^{-11}$–8.69×10^{-6}	3.65×10^{-10}–3.27×10^{-5}	$<10^{-11}$–9.04×10^{-6}	2.27×10^{-10}–5.45×10^{-6}
median	2.71×10^{-8}	6.84×10^{-5}	2.73×10^{-7}	8.53×10^{-6}

C_{max}, peak antibiotic concentration; C_{min}, minimum antibiotic concentration; ELF, epithelial lining fluid; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; PRU, prulifloxacin.

Results

MIC values

Basal MICs for the tested strains ranged between 0.5 and 2 mg/L for levofloxacin, 0.25 and 2 mg/L for ciprofloxacin, 0.06 and 0.25 mg/L for moxifloxacin and 0.5 and 1 mg/L for prulifloxacin.

Single-step selection of resistance

Mutational frequencies of *S. pneumoniae* for plasma and ELF fluoroquinolones concentrations are summarized in Table 2. Levofloxacin at 750 mg dosage and moxifloxacin were the drugs that best limited bacterial growth, with median frequencies of mutations below 10^{-11} both at plasma and ELF concentrations for all of the tested strains. Similar frequencies were also found for levofloxacin at 500 mg dosage at plasma and ELF C_{max}. Medians ranging from 10^{-8} to 10^{-6} were observed for ciprofloxacin and prulifloxacin at all concentrations. C_{min} for all the study drugs, except for levofloxacin at 750 mg dosage and moxifloxacin, were below the MIC values and therefore the tested strains were able to diffusely grow on the agar plate. For these strains, MICs of randomly sampled colonies were evaluated in order to detect any change in bacterial susceptibility.

Table 3 reports the MIC values for strains able to grow in the presence of the above-mentioned concentrations of all antimicrobials tested. Only two strains showed MIC increments of at least four times with respect to the basal values for 500 mg of levofloxacin at plasma C_{max} and C_{min} and at ELF C_{max}. Sustained increments were observed for ciprofloxacin with 10 and 12 strains with MICs four or more times the starting value at plasma and ELF concentrations, while marked changes in the MICs of prulifloxacin were observed less frequently (three, two, six and five strains at plasma C_{max} and C_{min} and ELF C_{max} and C_{min}, respectively).

Table 3. Fluoroquinolone activity against strains grown after single-step selection in *S. pneumoniae* ($n=20$) at plasma and ELF concentrations

Drug	MIC range (mg/L)/number of strains grown			
	C_{max} plasma	C_{min} plasma	C_{max} ELF	C_{min} ELF
LVX 500 mg	4/2	1–4/20	—/0	1–8/18
LVX 750 mg	—/0	—/0	—/0	—/0
MXF 400 mg	—/0	—/0	—/0	—/0
CIP 500 mg	4–8/12	1–4/20	2–16/18	1–4/20
PRU 600 mg	2–4 /11	0.25–2/20	2–4/18	0.5–4/20

C_{max}, peak antibiotic concentration; C_{min}, minimum antibiotic concentration; ELF, epithelial lining fluid; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; PRU, prulifloxacin.

Multi-step selection of resistant bacteria

Table 4 reports the total number of strains grown after multi-step selection and MIC values after 1, 5 and 10 passages on antibiotic-gradient plates and the following 10 passages on antibiotic-free medium. After multi-step selection, a general increase in MICs was observed for all microorganisms. This increase was rather unstable and most of the strains showed a decrease in MIC after 10 subcultures in antibiotic-free medium.

After 10 passages on antibiotic-gradient plates and 10 subcultures in antibiotic-free medium, the highest number of strains with MICs higher than the resistance breakpoint was found for

Table 4. MIC values after multi-step selection of resistance in *S. pneumoniae* ($n=20$)

Drug	Concentration	Number of strains grown	MIC (mg/L), median (range)				
			Pre-sel	I STEP	V STEP	X STEP	EP
LVX	plasma, 500 mg	9	2 (0.5–2)	2 (1–8)	4 (2–8)	8 (4–16)	4 (2–8)
	ELF, 500 mg	0	—	—	—	—	—
	plasma, 750 mg	3	2	2	4 (2–8)	16 (4–16)	4 (2–8)
	ELF 750 mg	0	—	—	—	—	—
MXF	plasma	0	—	—	—	—	—
	ELF	0	—	—	—	—	—
CIP	plasma	20	1 (0.25–2)	1 (1–4)	8 (4–16)	16 (8–32)	4 (2–16)
	ELF	16	1 (0.5–2)	4 (2–8)	8 (4–16)	16 (4–32)	2 (2–16)
PRU	plasma	20	1 (0.25–1)	4 (1–8)	8 (2–8)	1 (8–32)	8 (2–16)
	ELF	20	1 (0.25–1)	2 (1–8)	4 (2–16)	1 (8–32)	8 (2–32)

Pre-sel, starting MIC; I STEP, first subculture on an antibiotic-gradient plate; V STEP, fifth subculture on an antibiotic-gradient plate; X STEP, 10th subculture on an antibiotic-gradient plate; EP, 10th subculture on an antibiotic-free plate; ELF, epithelial lining fluid; CIP, ciprofloxacin; LVX, levofloxacin; MXF, moxifloxacin; PRU, prulifloxacin.

prulifloxacin and ciprofloxacin at plasma and ELF concentrations (19 and 31 strains, respectively), while levofloxacin at plasma concentrations selected for two resistant strains. No selection of resistance was observed for levofloxacin at ELF concentrations (both dosages) and for moxifloxacin at all concentrations (Table 4).

On the whole, plasma concentrations caused significant increments in MIC values (more than four times the baseline value) more frequently than ELF concentrations. Particularly, after 10 passages on antibiotic-gradient plates, the highest number of strains with a significant increase in the MIC was found for prulifloxacin and ciprofloxacin at plasma concentrations (19 and 17 strains, respectively) and ELF concentrations (19 and 11, respectively), while the lowest was observed for 750 mg of levofloxacin at plasma concentrations (2 strains) (Figure 1). Levofloxacin at ELF concentrations and moxifloxacin at plasma and ELF concentrations did not cause any significant increment in the MIC for the tested strains. After subculturing in antibiotic-free medium, a decrease in MIC occurred for all of the antibiotics tested, with prulifloxacin and ciprofloxacin still being the antibiotics with the highest number of strains with increased MIC (Table 4 and Figure 1).

Characterization of acquired resistance

Strains of *S. pneumoniae* selected by the multi-step assay and able to maintain their resistance after 10 passages in antibiotic-free medium were evaluated for acquired resistance. Among the 52 resistant mutants selected by fluoroquinolones, alterations in GyrA, ParC and ParE were found in 8, 40 and 14 mutants, respectively, while no mutations were found in GyrB. Mutations in GyrA, ParC and ParE are shown in Table 5. The two strains resistant to levofloxacin showed the classic substitution Ser79Phe in ParC; with regard to prulifloxacin and ciprofloxacin, the mutations identified were in GyrA (Ser81Phe), ParC (Ser79Phe; Ser79Tyr; Asp83Ala) and ParE (Ile460Val). Among prulifloxacin-resistant pneumococci, two mutants showed mutations in ParC, GyrA and ParE, three strains presented mutations in both ParC and GyrA and six strains presented mutations in both ParC and ParE. One ciprofloxacin-resistant mutant had mutations in ParC, GyrA and ParE, while mutations in ParC and GyrA were found in two mutants. Mutations in both ParC and ParE and in only ParC were shown by five and four ciprofloxacin-resistant mutants, respectively.

Discussion

As the frequency of both penicillin resistance and multidrug resistance is increasing among *S. pneumoniae*, respiratory fluoroquinolones, such as levofloxacin or moxifloxacin, have become the preferred drugs for the therapy of community-acquired respiratory tract infections, even though some concerns are now being raised about their widespread use and, possibly, misuse.[20] Despite its wide utilization in respiratory infections, it is doubtful whether ciprofloxacin is indicated for the treatment of CAP, as the MIC for *S. pneumoniae* is too close to concentrations obtained in bronchial secretion to guarantee efficacy.[25] Also, prulifloxacin shows intermediate activity against pneumococci and its concentration in ELF is near 1 mg/L, which is the tentative breakpoint for susceptibility indicated by the manufacturer. Despite this evidence, both ciprofloxacin and prulifloxacin are widely used in the therapy of community-acquired infections in clinical practice in Italy (IMS DATA 2008).

Several groups showed that the frequency of fluoroquinolone-resistant mutants is strain-dependent and decreases as the fluoroquinolone concentration is increased.[9,26,27] More importantly, it appears that the frequency of fluoroquinolone-resistant mutants is determined by the selective fluoroquinolone, the bacterial species and their load at the infection site.[9] Several reports have described the low frequency at which levofloxacin and moxifloxacin select for quinolone-resistant mutants of *S. pneumoniae*.[9,27–30]

In contrast to previous studies, the present study aimed to compare the ability to select for resistance in *S. pneumoniae* of levofloxacin, moxifloxacin, ciprofloxacin and prulifloxacin at concentrations equal to those found in plasma and ELF. The major advantage of this method resides in the use of a continuous gradient from peak to trough concentrations, which allowed us to assess simultaneously the effects of a wide range of

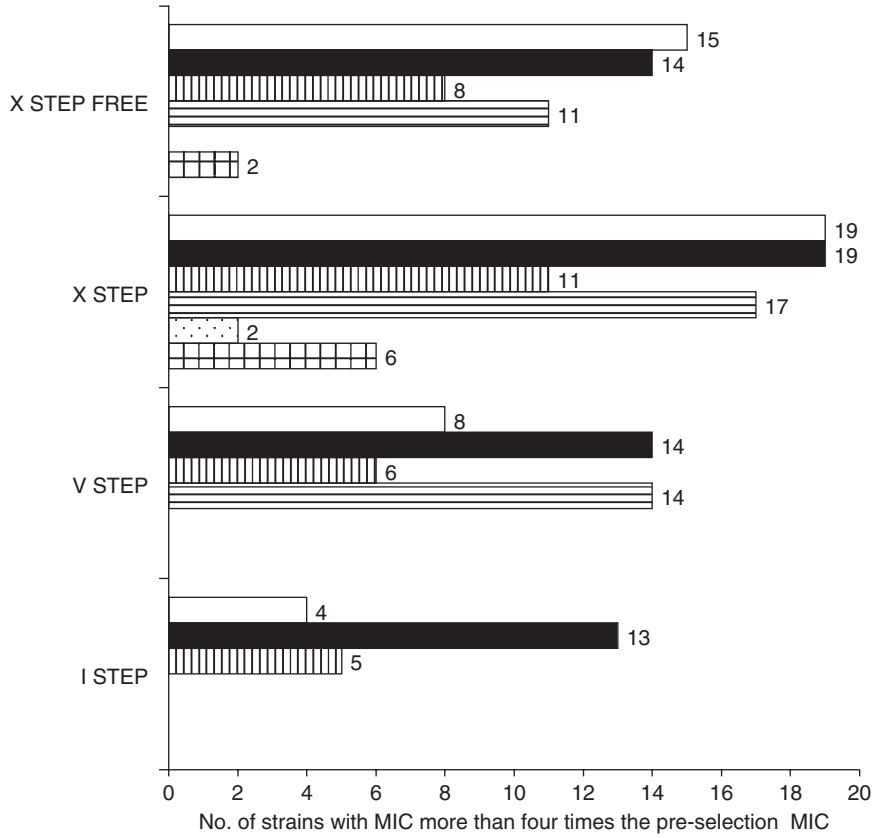

Figure 1. Multi-step selection of resistance in *S. pneumoniae*. Numbers of strains showing MIC increments of more than four times with respect to the baseline values during multi-step selection of resistance. White bars, prulifloxacin at ELF concentrations; black bars, prulifloxacin at plasma concentrations; bars with vertical lines, ciprofloxacin at ELF concentrations; bars with horizontal lines, ciprofloxacin at plasma concentrations; bars with dots, 750 mg of levofloxacin at plasma concentrations; bars with square grids, 500 mg of levofloxacin at plasma concentrations. I STEP, first subculture on an antibiotic-gradient plate; V STEP, fifth subculture on an antibiotic-gradient plate; X STEP, tenth subculture on an antibiotic-gradient plate; X STEP FREE, tenth subculture on an antibiotic-free plate.

Table 5. Amino acid changes encoded by mutations in *gyrA*, *gyrB*, *parC* and *parE*

	Replacement in QRDR (number of strains)			
Drug	GyrA	GyrB	ParC	ParE
LVX ($n=2$)	—	—	Ser79Phe (2)	—
CIP ($n=19$)	Ser81Phe (3)	—	Ser79Phe (12)	Ile460Val (6)
PRU ($n=31$)	Ser81Phe (5)	—	Ser79Phe (13), Ser79Tyr (2), Asp83Ala (11)	Ile460Val (8)

LVX, levofloxacin; CIP, ciprofloxacin; PRU, prulifloxacin.

concentrations usually occurring during therapy and that are generally considered as a target to reach in order to obtain the highest antibacterial efficacy.

Levofloxacin and moxifloxacin showed the lowest frequencies of mutations and a lower propensity than ciprofloxacin and prulifloxacin to select *in vitro* for resistance. Particularly, at pulmonary concentrations, our results showed a similar trend for levofloxacin and moxifloxacin, where both drugs did not select for resistance. In contrast, at plasma concentrations, mutations did not occur after exposure to moxifloxacin.

Ciprofloxacin is known to be associated with a high frequency of resistant mutants in *S. pneumoniae* and with a shorter time for emergence of decreased susceptibility.[9,26,31] Our study confirms these observations, also indicating that ciprofloxacin and prulifloxacin selected for resistance in a high number of strains, leading to a more marked decrease in susceptibility, with respect to levofloxacin and moxifloxacin.

It is possible that the similar efficacies observed for levofloxacin and moxifloxacin in preventing the development of resistance could be due to the higher serum and respiratory tissue

concentrations of levofloxacin, which likely offset the lower *in vitro* MIC for moxifloxacin.[31]

Decreased susceptibilities observed for colonies grown in single-step selection of resistance when the tested antibiotic concentrations fell below MIC suggest that development of resistance may occur in the absence of lethal antibiotic pressure. These data confirm the recent results of Avrain *et al.*,[9] who showed that subinhibitory concentrations of quinolones favour overexpression of efflux pumps or selection of target site mutations. This finding underlines the risk associated with the inappropriate use of antimicrobials, particularly when they are unable to reach optimal concentrations at the site of infection.

Alterations in *parC* and *gyrA* are generally considered the most frequent target gene mutations associated with fluoroquinolone resistance.[22–24] Positions in QRDRs evaluated in this study are known to be most frequently associated with resistance and have been identified in pneumococci isolated from respiratory infections that resulted in treatment failure associated with fluoroquinolone resistance.[8,23,32] In the present study, mutations in *parC* were found in 77% of mutants, while mutations in *parE* and *gyrA* were found in 27% and 15% of cases, respectively. The prevalence of mutations in *parC* was expected since topoisomerase IV is the preferred target of ciprofloxacin and prulifloxacin. Despite the fact that clinical isolates with mutations in *parC* are usually resistant to ciprofloxacin but susceptible to levofloxacin and moxifloxacin, they are known to be more likely to develop resistance to all quinolones during the therapy with the acquisition of a second-step *gyrA* mutation.[12] From this point of view, the high proportion of *parC* mutants selected by ciprofloxacin and prulifloxacin could suggest the risk of therapeutic failure associated with these agents, as previous exposure to these antibiotics could favour the development of resistance during subsequent treatment.

Interestingly, the two resistant mutants selected by levofloxacin, which derived from the same parental strain, presented mutations in *par*C only. Since, as mentioned earlier, levofloxacin resistance seems more frequently associated with mutations in both *parC* and *gyrA*, it is possible that mutations other than at positions Ser81, Glu85 and Ser114 could be involved, or that other mechanisms such as efflux pumps could have been selected.

For seven and five mutants, respectively, resistant to ciprofloxacin and prulifloxacin, no mutations in the QRDR of topoisomerase IV and gyrase were found. It is possible that resistance might be determined by mutations not corresponding to the hot spots analysed in the study or that other mechanisms might be responsible for resistance. A limitation of the present study is the lack of evaluation of the role of efflux in the development of resistance. Differential effects on efflux pump expression have been reported for moxifloxacin, levofloxacin and ciprofloxacin, the latter being a more potent inducer of efflux pump expression than levofloxacin, while moxifloxacin seems not to be affected by the efflux pumps observed in *S. pneumoniae*.[33,34]

In conclusion, levofloxacin and moxifloxacin, which are known to possess the highest activity against *S. pneumoniae* among the fluoroquinolones tested, are also characterized by a lower ability than ciprofloxacin and prulifloxacin to select *in vitro* for resistance when tested at concentrations occurring *in vivo*. Since the present study introduces a new approach to evaluating the capability to select for resistance, the potential clinical consequences of these findings remain to be estimated. New studies correlating the results obtained in this *in vitro* study to clinical data would be advisable.

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