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Selection of quinolone resistance in *Streptococcus pneumoniae* exposed *in vitro* to subinhibitory drug concentrations

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***Objectives*: Does exposure to subinhibitory concentrations of quinolones favour overexpression of efflux pumps or selection of target site mutations?**

***Methods*: ATCC 49619 (fully susceptible) and SP32 (clinical isolate with PmrA-mediated efflux and mutation in ParE) were exposed for 24 h in broth to ciprofloxacin, levofloxacin, moxifloxacin or garenoxacin at concentrations of 0.5× the MIC, with daily re-adjustments for up to 13 days. Efflux was detected phenotypically (decrease in MIC in the presence of reserpine), and expression of *pmrA* and *patA/patB* was measured by real-time PCR and comparative RT–PCR, respectively. Target site mutations were detected by sequencing of the quinolone resistance determining regions in *parC*, *parE* and *gyrA*. The clonal identity of isolates was checked by PFGE of genomic DNA.**

***Results*: Ciprofloxacin selected for stable mutants with 2.5–5-fold MIC increases for ciprofloxacin, 2–3-fold for levofloxacin and 1.3–2-fold for garenoxacin and moxifloxacin [partial reversion with reserpine for ciprofloxacin, gemifloxacin and levofloxacin (SP32 strain only), but not for garenoxacin and moxifloxacin]. Increased MICs were associated with overexpression of *patA/B* but not *pmrA*. In contrast, exposure to levofloxacin, moxifloxacin or garenoxacin selected target site mutations (*gyrA*, *parC*, *parE*) in both strains. Increases in MIC caused by efflux were similar to those caused by target site mutations.**

***Conclusions*: Exposure of *Streptococcus pneumoniae* to subinhibitory MICs of ciprofloxacin, a substrate for efflux pumps, results in *patA/B*-mediated efflux whatever the initial level of expression of *pmrA* of the strain. Quinolones that are poorly (levofloxacin) or not affected (moxifloxacin, garenoxacin) in their activity by efflux transporters preferentially select for target site mutants.**

Keywords: PmrA, PatA/PatB, reserpine, MIC

Introduction

Fluoroquinolones are bactericidal drugs that are highly effective in the treatment of *Streptococcus pneumoniae* infections.[1] Yet, they also appear to rapidly select for emergence of resistance.[2] This may lead to clinical failures[3–5] and creates a dilemma when selecting appropriate antibiotics for therapy at the level of the individual patient.[6] Analysis of resistance-related clinical failures with fluoroquinolones suggests that suboptimal treatments (resulting, in part, from the use of derivatives with inappropriate pharmacokinetic/pharmacodynamic profile) may be a key determinant.[7,8] Mutations in topoisomerases II and IV encoding genes, with *parC* and *gyrA* genes,[9,10] have been long considered as the most frequent mechanisms of resistance in *S. pneumoniae*. Yet, the role of efflux mechanisms[11–17] is now increasingly recognized as an important determinant in low to

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medium-level resistance towards fluoroquinolones.[13,18] Two types of fluoroquinolone efflux transporters have been reported so far. The first one belongs to the MFS (Major Facilitator Superfamily; energized by proton-gradients and called 'secondary transporters') and is encoded by *pmrA*.[15] The second class belongs to the ATP Binding Cassette superfamily (ABC, energized by ATP hydrolysis and called 'primary transporters'), with the two transporters identified so far encoded by *patA* and *patB*.[19]

Resistance through both target mutations and efflux following exposure of *S. pneumoniae* to subinhibitory concentrations of fluoroquinolones has already been observed,[20] but the level of expression of the corresponding transporters was not quantified. In the present work, we specifically tested for the ability of three fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) and one desfluoroquinolone (garenoxacin, because of its potential interest for use against *S. pneumoniae* isolates with resistance to other quinolones[21]) to select efflux-mediated resistance in *S. pneumoniae* towards these quinolones. The susceptibility of our strains to gemifloxacin, before and after exposure to the four quinolones mentioned above, was also examined in a later stage. To this end, we used in parallel a fully susceptible strain and a strain with increased expression of PmrA, and exposed them to subinhibitory concentrations of each of the above-mentioned quinolones. We determined the changes in both *pmrA* and in *patA* and *patB* expression and examined the occurrence of target site mutations, and correlated these changes with the increases of quinolone MICs over time.

Materials and methods

Bacterial strains and culture media

Two strains of *S. pneumoniae* were used in parallel, a wild-type strain without known resistance mechanism (ATCC 49619; ciprofloxacin MIC = 0.5 mg/L), obtained from the Institute for Medical Microbiology (Aachen, Germany) and a clinical isolate (SP32; nasal specimen obtained at the Cliniques Universitaires de Mont-Godinne, Yvoir, Belgium), with an elevated MIC of ciprofloxacin (1.5 mg/L) thought to be due to enhanced efflux, based on its return to wild-type susceptibility (MIC = 0.5 mg/L) in the presence of reserpine at a concentration of 10 mg/L. Both strains were grown in brain heart infusion (BHI) broth (Remel, Lenexa, KS, USA), or on Mueller–Hinton II agar (Becton–Dickinson France SAS, Le Pont-de-Claix, France) supplemented with 5% defibrinated horse blood (International Medical products s.a., Brussels, Belgium). Strains were stored at −80°C in BHI broth supplemented with 20% glycerol.

Antibiotics and efflux inhibitor (reserpine)

All antibiotics were obtained as microbiological standards (with >98% purity) from their respective manufacturers (with the potencies as indicated): ciprofloxacin (85%) and moxifloxacin (91%) from Bayer HealthCare (Leverkusen, Germany); levofloxacin (95%) from Aventis Pharma (Antony, France); garenoxacin (79%) from Toyama Chemical Co., Ltd (Toyama City, Japan); gemifloxacin (80%) from LG Life Sciences (Seoul, Korea). Stock solutions were prepared and stored as described previously[22] or following the manufacturer's recommendations (garenoxacin). Reserpine (Sigma-Aldrich, St Louis, MO, USA) was dissolved in dimethyl sulphoxide to a concentration of 2 mg/mL and thereafter diluted in the culture medium.

MIC determination

MICs were determined by arithmetic dilution in agar, using Mueller–Hinton medium supplemented with 5% defibrinated horse blood with or without reserpine (10 mg/L).[12,16,23–27] This agent is widely considered to be an efflux pump inhibitor[14,16,23,24] and so was used in the present study to reverse the resistance phenotype in *S. pneumoniae*.[15,28] The mechanism of this reversion remains, however, controversial.[28,29] Bacteria were grown in cation-adjusted Mueller–Hinton broth (Becton–Dickinson) supplemented with 3% lysed horse blood (Oxoid Ltd, Basingstoke, Hampshire, UK) to reach a density of 10^8 cfu/mL. One microlitre of this suspension was spotted with the help of a multipoint inoculator to the Petri dishes. Plates were read after 18 h of incubation at 37°C in air.

Resistance selection method

A serial passage method was used in which bacteria were grown in the continuous presence of a drug concentration corresponding to half of the MIC. The bacteria were examined daily for a change in MIC, followed by a corresponding increase of the drug concentration for up to 13 days. For this purpose, an initial inoculum of 2.5×10^6 cfu/mL of each of the original strains was exposed in broth to a range of antibiotic concentrations from 0.1- to 5–10-fold their original MIC (using arithmetic increases). After 24 h at 37°C, the tubes were examined to determine the minimal drug concentration preventing bacterial growth (this value was equal to the MIC determined on an agar plate for the corresponding strain). Bacteria growing at a drug concentration of half this value were then re-adjusted at a density of 2.5×10^6 cfu/mL, and again exposed for 24 h to drug concentrations from 0.1- to 5–10-fold the MIC. This process was repeated each day, looking for growth at drug concentrations larger than the original MIC. If this was observed, the new minimal drug concentration preventing bacterial growth was determined based on visual inspection of the cultures (and samples taken for confirmation of the MIC by agar dilution). Bacteria growing at a concentration corresponding to half of this new value were then used for continuation of the experiment, for a total of 13 days. All samples were kept for further analysis.

Strain characterization by PFGE

Genomic DNA was extracted and digested by *Sma*I enzyme as described previously.[30]

Extraction and purification of RNA and complementary DNA (cDNA) synthesis

S. pneumoniae was grown overnight at 37°C in 5% CO_2 on Mueller–Hinton agar supplemented with 5% horse blood. One colony was then grown at 37°C in 5% CO_2 in BHI broth until the end of log phase (absorbance at 620 nm of ~1 and 0.5 for ATCC 49619 and SP32, respectively). The cultures were chilled on ice and harvested by centrifugation at 5000 **g** for 5 min at 4°C. Pellets were stored for at least 30 min at −70°C. Samples were thawed, and total RNA was extracted using Qiagen RNeasy Columns (Qiagen Benelux B.V., Venlo, The Netherlands). Samples were then treated with turbo DNAseI (Ambion/Applied Biosystems, Austin, TX, USA). To check that all DNA had been removed, a PCR for the *parC* gene was performed. At least three RNA preparations were obtained independently from each strain. cDNA was synthesized from the RNA preparations using Superscript™ III H-reverse transcriptase (Invitrogen Ltd, UK) and random hexamer primers

(Invitrogen) according to the manufacturer's instructions and as previously described.[31]

pmrA gene expression by real-time PCR

Real-time PCR was performed in an iQ-cycler (Bio-Rad Laboratories, Hercules, CA, USA) with 25 μL of total reaction mixture containing 12.5 μL of iQ SYBR Green Supermix (2×), 400 nM forward pmrA-S (5′-TCCAGTATGGGCTTTTCCAG-3′) and reverse pmrA-AS (5′-CCAATCCAAAGAGGAAACGA-3′) primers and 5 μL of cDNA equivalent to 7.8 ng of reverse transcribed total RNA, in RNase/DNase-free water. *hexA* was used as a housekeeping gene to normalize levels of the *pmrA* transcripts.[32] Primer sequences for *hexA* were: forward hexA-S (5′-ACATTGAGCGCTTGGCTAGT-3′) and reverse hexA-AS (5′-ATCGCTGCGCTAATCAAACT-3′). Real-time cycling parameters were: one cycle at 95°C for 9.45 min followed by 40 cycles at 95°C for 15 s, 53.7°C for 1 min. The specificity of the real-time PCR was checked by a post-PCR melting-curve analysis performed under the following conditions: temperature starting at 60°C for 10 s followed by 0.5°C/10 s rises up to 95°C.

Expression of efflux pump genes patA and patB by comparative RT–PCR

The PCRs were carried out using a T-Personal PCR machine (Biometra/Whatman plc, Brentford, Middlesex, UK) with the following programme: 95°C for 5 min, then 30 cycles of 95°C for 1 min, 47°C for 1 min and 72°C for 1 min, followed by a final step of 72°C for 10 min. PCR was performed to amplify 16S rRNA gene, *patA* and *patB* from the cDNA. The PCR amplimers were quantified by DHPLC (denaturing high-performance liquid chromatography) as previously described[31] using the WAVE™ DNA fragment analysis system (Transgenomic Inc., Omaha, NE, USA). Within bacterial cells, the level of 16S rRNA was assumed to be constant throughout the growth conditions used in this study.[31,33] Variations in RNA preparations, which lead to differences in RT–PCR amplification levels, were normalized using the 16S rRNA levels as an internal control. The peak areas arising from Wave™ DHPLC analysis of each RT–PCR amplimer, for example *patA* or *patB*, were adjusted as necessary to compensate for variations between the mRNA levels in each RNA preparation.[31] Primer sequences for *patA* were: forward patA-S (5′-ATGTTGTCCTCGCAGCCTAT-3′) and reverse patA-AS (5′-ACGAACCGATGAACAAGAGG-3′); for *patB*: forward patB-S (5′-TTGCTGGTTCGGCTGTACTT-3′) and reverse patB-AS (5′-AACTGCTGTCATCTGGCCTT-3′); and for 16S RNA: forward 16S RNA-S (5′-GAGAAGAACGAGTGTGAGAG-3′) and reverse 16S RNA-AS (5′-CTAACACCTAGCACTCATCG-3′).

Mutation detections by sequencing

We searched for mutations in the quinolone resistance determining regions (QRDRs) of *parC*, *parE* and *gyrA*[16,24] (mutations in *gyrB* were not examined here because data from several independent studies strongly suggest that they contribute quite infrequently to clinically significant resistance[34–37]). These regions were amplified by PCR using primer sequences for *parC* as described previously;[10] for *parE*: forward parE-S (5′-CCAATCTAAGAATCCTG-3′) and reverse parE-AS (5′-GCAATATAGACATGACC-3′); and for *gyrA*: forward gyrA-S (5′-CCTGTTCACCGTCGCATTCT-3′) and reverse gyrA-AS (5′-AGTTGCTCCATTAACCA-3′). PCRs were performed at annealing temperatures 52, 54 and 49°C for *parC*, *parE* and *gyrA* genes, respectively, in 50 μL of mixture containing 200 μM each deoxynucleoside triphosphate, 250 nM each oligonucleotide primer, 2.5 U of *Taq* DNA polymerase in 1× of the corresponding buffer with 2 mM $MgCl_2$ and 2.5 μL of cDNA. Amplified products were purified by Qiaquick PCR purification kits (Qiagen) using the manufacturer's instructions, quantified and sequenced using the Big Dye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing conditions consisted of 30 cycles at 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

Sequences were analysed on a Genetic Analyser 3100 (Applied Biosystems). Sequencing was validated with control strains: PW 1698 (*gyrA*, Glu85Lys; *parC*, Ser79Phe; *parE*, Ile460Val), PW 1752 (*gyrA*, Glu85Gly; *parC*, Lys137Asn; *parE*, Asp435Asn), PW 1872 (*gyrA*, Ser81Tyr; *parC*, Lys137Asn; *parE*, Asp435Asn) and PW 1891 (*gyrA*, Ser81Phe; *parC*, Ser79Tyr).[38]

Results

MIC changes upon exposure to quinolones, effect of reserpine and occurrence of target mutations

Figure 1 shows the changes in MIC observed during the 13 days of exposure to half-MIC concentrations of ciprofloxacin. Considering ATCC 49619 first, there was a regular increase in MIC during the observation period, which was partially

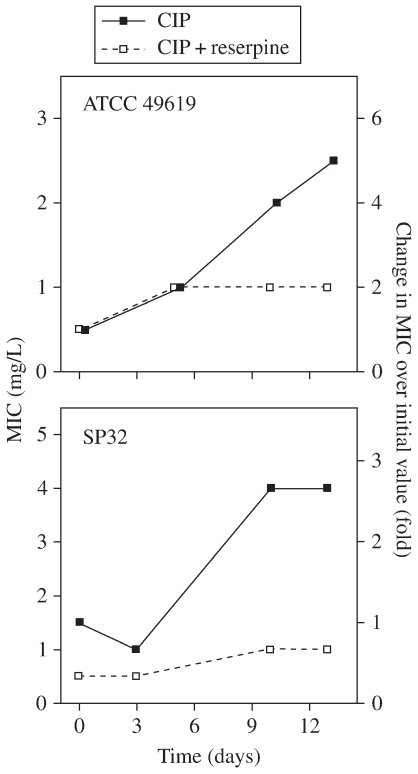

Figure 1. Evolution of the MIC of ciprofloxacin (CIP) for *S. pneumoniae* ATCC 49619 (upper panel) and SP32 (lower panel) after exposure to half-MIC concentrations of ciprofloxacin for the indicated times. MICs were determined in the absence (filled symbols and continuous lines) or in the presence (open symbols and broken lines) of 10 mg/L reserpine (the concentration of the antibiotic was re-adjusted each day to remain equivalent to half the MIC). MICs are plotted as actual values as measured by arithmetic dilutions (left-hand axis) or as multiples of the initial values as determined in the absence of reserpine (right-hand axis).

reversible upon reserpine addition after 5 days of exposure. SP32 was less susceptible to ciprofloxacin before starting the experiment, but reserpine decreased its MIC down to the level of ATCC 49619, suggesting that SP32 already expressed a fluoroquinolone efflux pump. As for ATCC 49619, there was a marked increase in MIC of ciprofloxacin during the observation period, which was almost completely reversed by addition of reserpine.

This experiment was repeated using moxifloxacin as the selecting agent, and alterations in the susceptibility of this population to both ciprofloxacin and moxifloxacin were investigated (Figure 2). For both fluoroquinolones, increases in MICs (10–27-fold in ATCC 49619 and 1.3–3.3-fold in SP32) were seen within 5–10 days. In contrast to what was seen after exposure to ciprofloxacin, increases in MIC after exposure to moxifloxacin were not reversed in the presence of reserpine.

To ascertain that the changes in MIC observed for either ciprofloxacin or moxifloxacin did not result from overgrowth of the original strains by contaminants, each sample was analysed by PFGE. The restriction patterns of DNA, which were different for ATCC 49619 and SP32, remained unmodified throughout the experiment (data not shown).

These experiments were then also repeated using levofloxacin and garenoxacin as selecting agents. All resistant mutants obtained during the resistance selection process with each quinolone were then examined for cross-resistance to other quinolones, and for occurrence of mutations in the QRDR of ParC, ParE and GyrA. Data are shown in Table 1, limited to the changes observed at the end of the exposure period (13 days, unless stated otherwise). Considering ATCC 49619 first, exposure to ciprofloxacin mainly affected susceptibility to ciprofloxacin itself (5-fold increase in MIC), to a lesser extent to levofloxacin (3-fold increase in MIC), and only modestly to moxifloxacin, garenoxacin and gemifloxacin ($\leq$2-fold increase in MIC), demonstrating a certain level of dissociated resistance. Reserpine reduced the MIC of ciprofloxacin only. When levofloxacin, moxifloxacin or garenoxacin were used as selecting agents, 2–27-fold increases in MIC values were seen for all quinolones. Reserpine had no effect on these changes in MICs. However, target mutations were observed in *GyrA* (Ser81) when levofloxacin was used as a selecting agent, and in both *GyrA* (Ser81) and *ParC* (Ser79) when moxifloxacin and garenoxacin were used (explaining the higher increases in MIC seen with these antibiotics).

The clinical strain (SP32) initially had a mutation within *ParE* (Ile460Val). Exposure to ciprofloxacin caused a marked increase in ciprofloxacin MIC and a more modest ($\leq$2-fold) increase in levofloxacin and gemifloxacin MICs, all partly reversed by reserpine. No additional mutation was seen. When levofloxacin was used as a selecting agent, 2–6-fold increases in MICs were seen for all quinolones. The increases in MICs were associated with an additional mutation in the QRDR of *parC*, with no or only modest effect of reserpine. When moxifloxacin and garenoxacin were used as selecting agents, 2–3-fold increases in MICs were seen, with only a modest effect of reserpine, but with supplemental mutations in *parE* after moxifloxacin exposure.

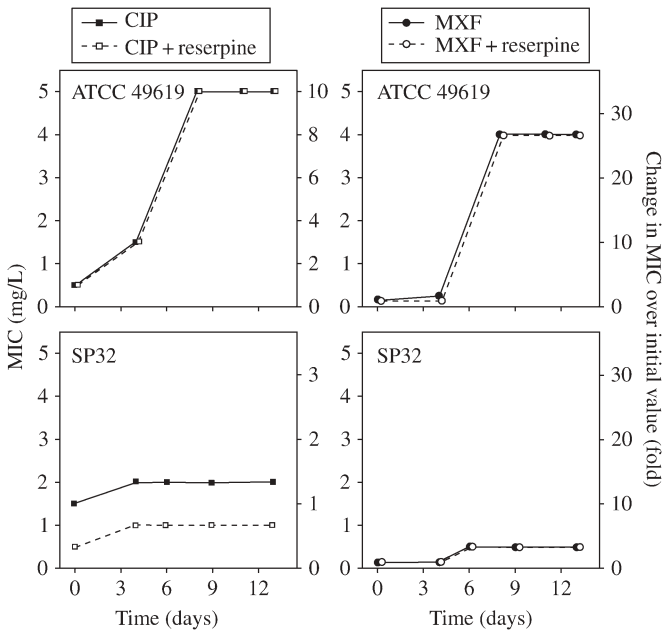

Figure 2. Evolution of the MIC of ciprofloxacin (CIP; left-hand panels) and of moxifloxacin (MXF; right-hand panels) for *S. pneumoniae* ATCC 49619 (top panels) and SP32 (bottom panels) after exposure to half-MIC concentrations of moxifloxacin for the indicated times. MICs were determined in the absence (filled symbols and continuous lines) or in the presence (open symbols and broken lines) of 10 mg/L reserpine and the concentration of the inducer antibiotic was re-adjusted each day to remain equivalent to half the MIC. MICs are plotted as actual values as measured by arithmetic dilutions (left-hand axis) or as multiples of the initial values as determined in the absence of reserpine (right-hand axis).

pmrA and patA/patB expression levels

The expression of the genes encoding the two types of quinolone efflux transporters (PmrA and PatA/PatB) was determined in both sets of strains, i.e. before antibiotic exposure and after 13 days of culture. We focused our efforts on bacteria exposed to ciprofloxacin, since these were the most likely to harbour this new efflux-mediated mechanism of resistance, while using bacteria exposed to moxifloxacin as a potential negative control. Data are shown in Figure 3. Initially, *pmrA* expression was markedly higher in SP32 (11-fold higher) than in ATCC 49619, whereas *patA/patB* expression level was similar in both strains. In mutants of SP32 obtained after exposure to ciprofloxacin there was a decrease in *pmrA* expression. For mutants from both strains, the expression of *patA* and *patB* was significantly increased after ciprofloxacin exposure. Exposure to moxifloxacin did not affect the expression level of the three genes investigated.

Discussion

The present study shows that exposure of *S. pneumoniae* to subinhibitory concentrations of quinolones caused a decrease in susceptibility to ciprofloxacin, levofloxacin, garenoxacin, moxifloxacin and gemifloxacin, the mechanism of which differed depending on the antibiotic used for selection rather than on the initial phenotype of the strain. Two resistance mechanisms were observed, active efflux and target site mutations (topoisomerases II and IV). The changes in MIC observed were

Table 1. Initial and final (at day 13) MICs of ciprofloxacin (CIP), levofloxacin (LVX), moxifloxacin (MXF), garenoxacin (GRN) and gemifloxacin (GMF) for ATCC 49619 and SP32 strains, measured in the absence or presence of reserpine (res), and nature of the mutations detected in the QRDR domains (*parC*, *parE*, *gyrA*) of topoisomerases II and IV (sequencing)

		MIC (mg/L)									
				after selection[b] with							
		initial[a]		CIP		LVX		MXF		GRN	
Strain	Drug	res −	res +	res −	res +	res −	res +	res −	res +	res −	res +
ATCC 49619	CIP	0.5	0.5	2.5	**1**	1.5	1.5	5[c]	5[c]	>4	>4
	LVX	0.5	0.5	1.5	1.5	2.5	2.5	4[c]	4[c]	4	4
	MXF	0.15	0.15	0.25	0.25	1	1	4[c]	4[c]	4	4
	GRN	0.05	0.05	0.1	0.1	0.25	0.25	1[c]	1[c]	2	2
	GMF	0.05	0.05	0.08	**0.05**	0.1	0.1	0.3[c]	0.3[c]	0.3	0.3
		—		—		GyrA (Ser81Phe)		GyrA (Ser81Phe)[c] ParC (Ser79Tyr)[c]		GyrA (Ser81Tyr) ParC (Ser79Tyr)	
SP32	CIP	1.5	**0.5**	4	**1**	4[c]	**3**[c]	2	**1**	1.5	**1**
	LVX	0.75	0.75	1.5	**1**	2.5[c]	**2**[c]	2.5	**2**	1.5	1.5
	MXF	0.15	0.15	0.2	0.2	0.6[c]	0.6[c]	0.5	0.5	0.4	0.4
	GRN	0.05	0.05	0.1	0.1	0.1[c]	0.1[c]	0.05	0.05	0.2	0.2
	GMF	0.03	0.03	0.06	**0.04**	0.1[c]	**0.05**[c]	0.1	0.1	0.1	0.1
		ParE (Ile460Val)		ParE (Ile460Val)		ParE (Ile460Val) ParC (Ser79Phe)[c]		ParE (Ile460Val) ParE (Arg447Cys)		ParE (Ile460Val)	

Bold text indicates conditions in which MIC is reduced by addition of reserpine.
[a]MIC (mg/L) before exposure to antibiotics.
[b]MIC (mg/L) after 13 days of exposure to re-adjusted half MICs of each of the antibiotics studied inducer (see the Materials and methods section for details), unless stated otherwise.
[c]MICs or resistance mechanism observed at day 10 (LVX) or 11 (MXF).

both significant and reproducible, based on (i) the use of arithmetic dilutions (allowing detection of minor changes in susceptibility) and (ii) the comparison between the value observed in broth during the selection process and that measured independently on agar. Before selection, ATCC 49619 displayed the expected susceptibility levels based on previous published data;[39,40] similarly SP32 displayed the expected susceptibility level based on the presence of an efflux mechanism and first-step mutation in *parE*.[13,41] The magnitude of the changes seen during the selection process was in all cases moderate, and in principle compatible with the known mechanisms of resistance of *S. pneumoniae* towards quinolones.[9,12,15,34]

Resistance associated with exposure to ciprofloxacin was mediated by increased efflux (based on the reversibility of the resistance phenotype in the presence of reserpine), both in the wild-type ATCC 49619 and in SP32 which overexpresses the gene coding for the PmrA efflux transporter. Interestingly, this was due to significantly increased expression of *patA/patB*, but not of *pmrA* (even in the SP32 strain that already overexpressed this transporter). Assuming that reserpine interacts with both PmrA and/or PatA and PatB, the data suggest differences in substrate specificities for fluoroquinolones, since only ciprofloxacin showed a reserpine-reversible MIC elevation in the *pmrA*-overexpresser SP32, while both ciprofloxacin and levofloxacin, and to a lesser extent gemifloxacin, displayed reserpine synergy in the *patA/patB* overexpressers selected by ciprofloxacin. These data, therefore, support the concept that moxifloxacin and garenoxacin are less likely to be affected, and gemifloxacin and levofloxacin only modestly affected in their activity by the efflux pumps observed in *S. pneumoniae*.[18] They also further confirm the proposal that PmrA is probably not the main transporter involved in quinolone efflux in *S. pneumoniae*.[25,26,42–44]

In contrast to ciprofloxacin, levofloxacin, moxifloxacin and garenoxacin did not select for efflux, as also demonstrated in other *in vitro* and *in vivo* models.[23,27] We tentatively interpret this observation as a further indication that these drugs are only partially or not recognized by the transporters described so far, resulting probably from the difference in the hydrophilic/hydrophobic balance between ciprofloxacin and other quinolones.[23] The behaviour of levofloxacin (subject to efflux but not selecting for it) is of special interest since it may help in differentiating which determinants in a fluoroquinolone molecule make it a substrate and which ones trigger the overexpression of the corresponding transporter (unfortunately, selection of resistance by exposure to gemifloxacin, a poor substrate of efflux transporters, could not be examined in the present study). Being partly (e.g. levofloxacin) or fully (e.g. moxifloxacin) refractive to efflux, however, did not protect against resistance selection, since this was easily obtained by mutation (single or dual) in the QRDRs, resulting in a cross-resistant phenotype, as previously reported[20,45] (it could even be suggested that lack of efflux favours selection of resistance through target mutation, but this requires further investigation). In this context, our study also confirms that dual mutations in both *parC* and *gyrA* genes cause a larger decrease in susceptibility compared with mutations in one gene only, as already reported.[9,10]

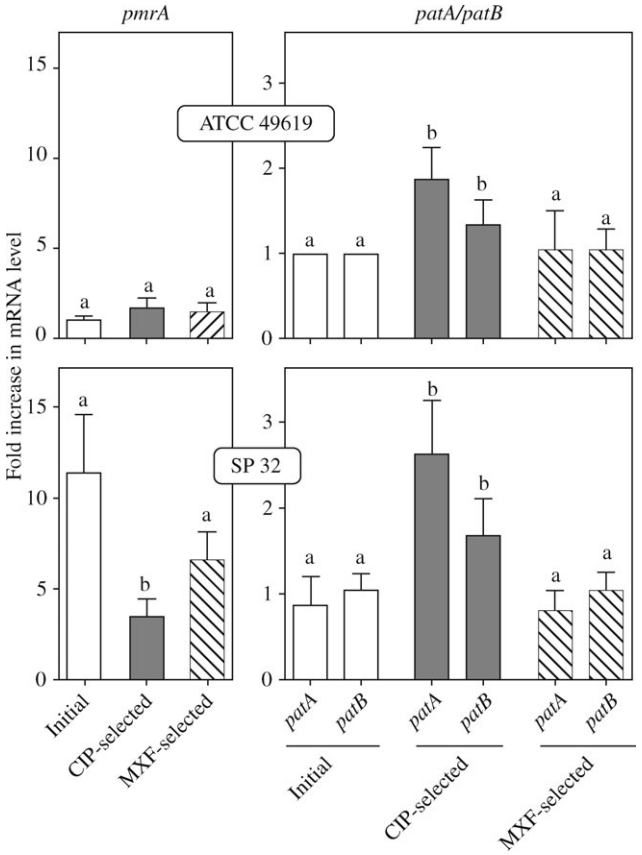

Figure 3. Expression levels of *pmrA* (left-hand panels) and *patA* and *patB* (right-hand panels) in ATCC 49619 (top panels) and SP32 (bottom panels) before selection and after 13 days of exposure to ciprofloxacin (CIP) or moxifloxacin (MXF) at half their MICs. Data are expressed in multiples of the value measured for the strain ATCC 49619 before exposure to antibiotics. Values are means ± SD of three RNA preparations with one RT–PCR for each RNA preparation. Statistical analysis (two-tailed Student's *t*-test comparing initial level of expression of a given gene with expression levels in resistant strains selected by antibiotics): bars with different letters (b versus a) are significantly different from one another ($P < 0.05$).

Two important observations from our study need to be underlined. First, we see that efflux-mediated resistance may cause a decrease in susceptibility that is almost as great as that conferred by target site mutations. Moreover for ciprofloxacin, it may develop in the absence of mutation suggesting this mechanism is both a first and sufficient line of defence against hydrophilic fluoroquinolones. This means that the widely accepted concept that efflux causes only low-level resistance (as opposed to target mutations) may need to be revisited. Thus, we show that efflux may increase the MIC to the limits of susceptibility set by PK-PD-based considerations for efficacy as well as prevention of emergence of resistance.[21] The potential clinical consequences should not be underestimated, since the majority of these strains would still be recorded as fully susceptible when using the breakpoint criteria defined by the CLSI (formerly NCCLS) (http://www.clsi.org/). In contrast, these strains will be considered as not '*fulfilling conditions for obtaining a high likelihood of therapeutic success*' based on the definition of susceptibility proposed by the European Committee for Antibiotic Susceptibility Testing (EUCAST; http://www.eucast.org). This points out the necessity of regularly revisiting commonly accepted breakpoints, or, better, to rely, as far as possible, on true MIC measurements and positive determination of the occurrence of efflux mechanisms in epidemiological surveys as well as in difficult clinical situations. Our study indicates, indeed, that such strains can readily develop and therefore need to be properly identified. An analysis of a much larger number of isolates than those tested in the present study would be interesting to perform in this context. Second, we see that development of resistance may occur in the absence of lethal antibiotic pressure (since our experiments systematically used an exposure to 0.5× MIC for selection). This clearly points to the potential adverse effects of sub-MIC concentrations and underlines the risk of treating patients sub-optimally with quinolones. This is of particular importance for molecules with weak potency against their target organism.[5,46] Conversely, the fact that prolonged exposure to quinolones is needed to select resistance may be an argument for promoting short therapy courses, coupled with conditions ensuring appropriate drug exposure during therapy.

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Transparency declarations

None to declare.

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