In Vitro Development of Resistance to Six Quinolones in *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*

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***Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus* isolates were exposed to subinhibitory MICs of ciprofloxacin, sparfloxacin, gatifloxacin, moxifloxacin, clinafloxacin, and gemifloxacin during a 10-day period. Subculturing led to resistance development, regardless of the initial potencies of the quinolones. None of the quinolones was associated with a significantly slower rate of resistance development.**

Fluoroquinolone resistance in gram-positive cocci is related to mutations in the DNA gyrase and topoisomerase IV genes (8–12, 16, 23) and the active efflux of agents (1, 3, 13, 21–25, 31, 44). Because fluoroquinolones differ in both their target affinity (8, 16, 33–36, 39) and their activation of efflux pumps (7, 13, 21, 22, 24, 25, 31, 42), one can speculate that the phenotypic expression of quinolone resistance will also differ. Studies have shown that fluoroquinolone resistance can be selected for in pneumococci and staphylococci (5, 6, 37).

In order to analyze the ability of newer fluoroquinolones to cause resistance development in *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Staphylococcus aureus*, we repeatedly exposed six clinical strains of each species to ciprofloxacin, sparfloxacin, gatifloxacin, moxifloxacin, clinafloxacin, and gemifloxacin.

Approximately 5×10^7 CFU of each of the 18 strains was added to tubes containing 9.9 ml of appropriate broth containing antibiotic concentrations ranging from 3 doubling dilutions above to 3 doubling dilutions below the MIC of each of the six agents. The tubes were then incubated for 24 h at 37°C. Aliquots from the test tubes containing the highest drug concentration that permitted visible growth were used following a 1:100 dilution to inoculate a second set of serial drug dilutions. After overnight incubation, the bacteria were transferred again. Finally, after 10 serial transfers, the bacteria for which the MICs were the highest were collected, stored, and also subcultured on quinolone-free agar for 10 days to assess the stability of resistance.

MICs were determined by the microdilution methodology according to NCCLS guidelines (29, 30). Ciprofloxacin MIC

determinations were conducted in the presence and absence of reserpine (20 µg/ml; tests were repeated three times) for all of the original isolates ($n = 18$) as well as for all of the selected mutants ($n = 108$) (7).

S. pneumoniae and *S. aureus* isolates were analyzed before and after transfers for mutations in the quinolone-resistance determining regions (QRDRs) of *parC* or *grlA* and *gyrA*, respectively (19, 40, 41).

The MIC results from subculturing as well as the mutations in the QRDRs of *S. pneumoniae* and *S. aureus* are summarized in the Tables 1 to 3. Subculturing with newer quinolones led to resistance development in all three species. This is in line with previous reports with regard to cephalosporins, macrolides, and older quinolones in pneumococci (5, 6, 37).

Resistance was stable in all cases; i.e., the MICs for the 108 selected mutants remained within 1 doubling dilution after 10 transfers on quinolone-free agar. Similar results were reported by Davies et al. (5) for *S. pneumoniae*.

Gemifloxacin and clinafloxacin exhibited the best in vitro activities against all original isolates, followed by moxifloxacin and gatifloxacin. Ciprofloxacin showed the lowest in vitro activities (Tables 1 to 3). On the basis of a breakpoint of ≤ 1 µg/ml, 88 of the 108 selected mutants were inhibited by clinafloxacin, 65 were inhibited by gemifloxacin, 46 were inhibited by moxifloxacin, 32 were inhibited by gatifloxacin, 20 were inhibited by sparfloxacin, and 3 were inhibited by ciprofloxacin. This rank order of activity is in parallel with previous findings (2, 4, 7, 19, 20, 26).

On the basis of the results presented in Tables 1 to 3, none of the quinolones was associated with a clearly lower potential for resistance development, measured as an increase in MIC doubling dilutions, compared with the potential for resistance development of the other quinolones tested. On the basis of the limited available data, one must be cautious in drawing broad conclusions. Further studies will be needed to

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TABLE 1. Resistance selection results for *S. pneumoniae*^a

Strain	Initial MIC (μg/ml)						Selected resistance		MIC (μg/ml) upon retesting						Alteration ^c in enzymes of:	
	CIP	SPX	GAT	MOX	CLI	GEM	Drug	Fold increase in MICs ^b	CIP	SPX	GAT	MOX	CLI	GEM	GyrA	ParC
1	0.25	0.125	0.125	0.06	0.03	0.015	CIP	8	64	8	8	2	0.5	0.5	S81F	S79Y
							SPX	7	32	16	8	2	0.25	0.5	S81Y	S79Y
							GAT	8	32	32	32	16	8	1	S81F	S79Y
							MOX	10	64	32	64	64	8	8	S81F	S79Y, D83N
							CLI	5	64	16	8	4	1	2	S81F	S79Y
							GEM	9	32	32	128	32	8	8	S81F	S79Y
2	0.25	0.06	0.06	0.03	0.03	0.015	CIP	5	8	0.125	0.5	0.06	0.25	0.125	S81F	
							SPX	5	4	2	1	0.5	0.125	0.125	S81F	
							GAT	4	4	0.5	1	0.5	0.25	0.125	S81F	
							MOX	5	16	4	4	1	0.5	0.25	S81F	
							CLI	4	4	2	1	0.5	0.5	0.125	S81Y	
							GEM	7	32	8	64	2	2	2		D83Y
3	1	0.125	0.25	0.125	0.125	0.015	CIP	6	64	8	8	2	1	0.5	S81F	S79Y, D83G
							SPX	6	32	8	4	2	0.5	0.25	S81F	D83G
							GAT	5	32	16	8	4	1	0.5	S81F	D83G
							MOX	5	64	32	16	4	2	2	S81F	S79Y
							CLI	3	32	8	4	2	1	0.5	S81F	D83N
							GEM	6	16	4	64	1	2	1	S81F	
4	1	0.125	0.25	0.125	0.06	0.015	CIP	6	64	8	8	2	1	0.5	S81F	S79Y, D83G
							SPX	6	8	8	4	2	0.5	0.25	S81F	
							GAT	3	8	4	2	1	0.5	0.25	S81F	
							MOX	6	64	32	16	8	1	1	S81F	S79F
							CLI	3	4	2	2	1	0.5	0.25	S81F	
							GEM	8	16	4	64	2	1	4	S81F	S79Y
5	1	0.125	0.25	0.125	0.06	0.015	CIP	1	2	0.25	0.5	0.25	0.125	0.06		
							SPX	7	8	16	4	4	0.5	0.5	S81F	S79F
							GAT	4	16	32	4	4	0.5	0.5	S81Y	S79F
							MOX	4	8	8	4	2	0.25	0.25	S81F	
							CLI	3	32	16	8	4	0.5	0.5	S81Y	S79F
							GEM	6	32	8	32	1	0.5	1		D83Y
6	0.25	0.03	0.06	0.03	0.03	0.015	CIP	8	64	4	4	2	0.5	0.5	S81F	S79Y
							SPX	5	2	1	0.5	0.5	0.125	0.06		
							GAT	10	8	8	64	16	4	2	S81F	S79F
							MOX	7	8	2	4	4	0.25	0.125	S81F	S79A
							CLI	4	2	1	1	0.5	0.5	0.03	S81Y	
							GEM	4	8	1	32	1	0.125	0.25	S81F	

^a CIP, ciprofloxacin; SPX, sparfloxacin; GAT, gatfloxacin; MOX, moxifloxacin; CLI, clinafloxacin; GEM, gemifloxacin.^b Fold increase in MIC between the MICs for the quinolone-resistant strain selected in the quinolone-containing medium and the MIC for the parental strain. For example, the ciprofloxacin MIC for parent strain 1 was 0.25 μg/ml. After passages in ciprofloxacin-containing medium, the ciprofloxacin MIC had increased to 64 μg/ml, i.e., an eightfold increase.^c Alterations refer to changes between the parent strain and the derived strain. All parent strains had wild-type ParC and GyrA enzymes.

clarify whether resistance development is strain dependent or dichotomous, especially for the 8-methoxyfluoroquinolones (gatfloxacin, moxifloxacin) compared with the other compounds.

For five of the six original *S. pneumoniae* strains tested, ciprofloxacin MICs were 1 to 3 doubling dilutions lower in the presence of reserpine. After 10 serial passages in ciprofloxacin-containing medium, the ciprofloxacin MICs for the same five of the six *S. pneumoniae* mutants were again 1 to 3 doubling dilutions lower in the presence of reserpine than in its absence. The MICs for five of six of the mutants selected with moxifloxacin or clinafloxacin were 1 to 2 doubling dilutions lower, the MICs for five of six of the mutants selected

with sparfloxacin or gatfloxacin were 1 to 3 doubling dilutions lower, and finally, the MICs for five of six of the mutants selected with gemifloxacin were 2 to 4 doubling dilutions lower.

Ciprofloxacin MICs for none of the six original *S. pyogenes* strains tested were lower in the presence of reserpine. After 10 serial passages, the ciprofloxacin MICs for only one mutant selected with gemifloxacin were decreased by 3 doubling dilutions.

Ciprofloxacin MICs for none of the six original *S. aureus* strains tested were lower in the presence of reserpine. After 10 serial passages, ciprofloxacin MICs for six of six mutants selected with ciprofloxacin were 2 to 3 doubling dilutions lower in

TABLE 2. Resistance selection results for *S. pyogenes*^a

Strain	Initial MIC ($\mu\text{g/ml}$)						Selected resistance		MIC ($\mu\text{g/ml}$) upon retesting					
	CIP	SPX	GAT	MOX	CLI	GEM	Drug	Fold increase in MICs ^b	CIP	SPX	GAT	MOX	CLI	GEM
1	0.5	0.25	0.25	0.125	0.03	0.03	CIP	2	2	1	1	0.5	0.125	0.06
							SPX	5	4	8	1	0.5	0.125	0.25
							GAT	3	2	4	2	1	0.25	0.125
							MOX	3	2	4	1	1	0.25	0.125
							CLI	3	1	1	0.5	0.5	0.25	0.06
							GEM	3	2	4	1	2	0.25	0.25
2	0.125	0.06	0.06	0.06	0.03	0.06	CIP	7	16	16	4	2	0.5	0.25
							SPX	9	16	32	16	8	2	2
							GAT	6	16	16	4	4	0.5	0.5
							MOX	8	16	32	32	16	4	4
							CLI	4	8	8	4	2	0.5	0.125
							GEM	3	8	4	2	2	0.25	0.5
3	2	0.5	0.25	0.25	0.06	0.06	CIP	2	8	4	2	2	0.5	0.125
							SPX	4	8	8	4	2	0.5	0.5
							GAT	4	16	8	4	2	1	0.25
							MOX	6	32	64	16	16	2	2
							CLI	6	32	64	32	16	4	1
							GEM	5	16	32	4	8	0.5	2
4	0.5	0.25	0.25	0.125	0.06	0.03	CIP	6	32	32	4	4	0.5	0.5
							SPX	7	16	32	4	4	0.5	0.5
							GAT	2	2	2	1	1	0.25	0.25
							MOX	3	2	2	1	1	0.125	0.125
							CLI	1	2	1	0.5	0.5	0.125	0.06
							GEM	6	16	16	8	8	2	2
5	0.5	0.25	0.25	0.125	0.03	0.03	CIP	2	2	2	1	1	0.25	0.06
							SPX	4	4	4	4	1	0.25	0.25
							GAT	2	2	4	1	1	0.125	0.25
							MOX	4	4	16	2	2	0.25	1
							CLI	2	1	1	0.5	0.25	0.125	0.125
							GEM	3	4	2	1	1	0.25	0.25
6	0.5	0.5	0.5	0.25	0.03	0.03	CIP	3	4	1	1	0.5	0.25	0.06
							SPX	4	8	8	1	1	0.25	0.25
							GAT	2	4	2	2	1	0.25	0.125
							MOX	3	4	2	2	2	0.25	0.125
							CLI	3	4	4	2	1	0.25	0.25
							GEM	6	16	32	8	8	2	2

^a CIP, ciprofloxacin; SPX, sparfloxacin; GAT, gatifloxacin; MOX, moxifloxacin; CLI, clinafloxacin; GEM, gemifloxacin.

^b Fold increase in MICs between the MIC for the quinolone-resistant strain selected in the quinolone-containing medium and the MIC for the parental strain. For example, the ciprofloxacin MIC for parent strain 1 was 0.5 $\mu\text{g/ml}$. After passages in ciprofloxacin-containing medium, the ciprofloxacin MIC had increased to 2 $\mu\text{g/ml}$, i.e., a 2 twofold increase.

the presence of reserpine, MICs for four of six mutants selected with sparfloxacin or gatifloxacin were 1 doubling dilution lower, MICs for three of six mutants selected with clinafloxacin were 2 doubling dilutions lower, and, finally, MICs for six of six mutants selected with gemifloxacin were 2 to 4 doubling dilutions lower.

These results illustrate the importance of efflux as an additional factor for the development of quinolone resistance in *S. aureus*, followed by *S. pneumoniae*, while efflux seems not to play a significant role in the process of quinolone resistance development in *S. pyogenes*. Furthermore, our data indicate that some of the newer fluoroquinolones, especially gemifloxacin, selected mutants which had reserpine-sensitive phenotypes for ciprofloxacin resistance. This phenomenon must be kept in mind during therapy with newer quinolones.

Mainly classical alterations in ParC (a Ser-79→Phe or Tyr or Asp-83→Asp) and GyrA (a Ser-81→Phe or Tyr) contributed to the resistance seen in most *S. pneumoniae* mutants (Table 1). These results confirm those from previous investigations (12, 15, 17, 19, 20, 27, 32, 38, 43).

In addition, mainly classical alterations in GrlA (a Ser-80→Phe or Tyr, a Glu-84→Lys) and GyrA (a Ser-84→Leu or Lys and a Glu-88→Lys or Val) contributed to the resistance in most *S. aureus* mutants (Table 3). These results are in line with previous observations (14, 18, 28, 40, 41).

In summary, sequential subculture in the presence of subinhibitory concentrations of newer fluoroquinolones led to resistance development in *S. pneumoniae*, *S. pyogenes*, and *S. aureus*. None of the quinolones tested was associated with a clearly lower potential of resistance development.

TABLE 3. Resistance selection for *S. aureus*^a

Strain	Initial MIC (μg/ml)						Selected resistance		MIC (μg/ml) upon retesting						Alterations ^c in enzymes of:	
	CIP	SPX	GAT	MOX	CLI	GEM	Drug	Fold increase in MICs ^b	CIP	SPX	GAT	MOX	CLI	GEM	GyrA	GrlA
1	0.25	0.06	0.06	0.06	0.015	0.015	CIP	7	32	8	4	2	0.5	2	S84L	E84K
							SPX	8	64	16	4	4	1	0.5	S84L	S80Y
							GAT	6	64	16	4	8	0.5	4	S84L	E84K
							MOX	3	4	0.5	1	0.5	0.25	0.015	S84A	S80F
							CLI	7	128	8	16	2	2	4	S84L	E84K
							GEM	13	2,048	64	128	32	32	128	S84L	S80F
2	0.25	0.06	0.06	0.06	0.03	0.015	CIP	6	16	8	4	1	0.5	0.06	S84L	E84K
							SPX	6	4	4	2	1	0.25	0.5	S84L	S80F
							GAT	4	8	0.5	1	0.5	0.125	0.25	E88G	S80F
							MOX	4	4	4	1	1	0.25	0.5	S84L	E84K
							CLI	4	16	8	4	1	0.5	0.5	E88K	E84K
							GEM	3	16	1	4	4	0.5	0.125	S84L	S80F
3	0.25	0.06	0.06	0.06	0.03	0.015	CIP	5	8	0.125	0.5	0.125	0.125	0.25	S84L	E84K
							SPX	6	4	4	2	1	0.125	0.5	S84L	S80Y
							GAT	4	8	4	1	1	0.25	0.5	S84L	S80Y
							MOX	3	4	4	2	0.5	0.125	0.5	S84L	E84K
							CLI	4	16	8	4	2	0.5	2	S84L	S80F
							GEM	9	128	8	8	4	0.5	8	S84L	E84K
4	0.125	0.06	0.06	0.06	0.03	0.03	CIP	11	256	1	8	1	2	2	S84L	S80F
							SPX	8	32	16	16	2	0.5	2	S84L	E84K
							GAT	11	128	128	128	32	8	16	S84L	S80Y
							MOX	10	64	128	128	64	16	16	E88K	S80Y
							CLI	5	128	64	4	1	1	1	S84L	S80F
							GEM	6	16	1	1	8	0.25	1	S84L	S80Y
5	0.25	0.06	0.06	0.06	0.03	0.015	CIP	8	64	8	8	4	1	2	S84L	S80Y
							SPX	8	32	16	4	4	1	2	S84L	S80Y
							GAT	7	32	16	8	4	1	2	S84L	S80Y
							MOX	6	64	16	4	4	1	2	S84L	S80Y
							CLI	6	64	16	4	4	2	2	S84L	E84K
							GEM	12	1,024	32	32	16	8	64	S84L	S80Y
6	0.25	0.06	0.06	0.06	0.03	0.015	CIP	8	64	8	8	2	1	4	S84L	E84G
							SPX	2	0.5	0.25	0.12	0.125	0.03	0.06		S80Y
							GAT	4	4	0.5	1	0.25	0.125	0.06		S80F
							MOXI	0	0.25	0.06	0.06	0.06	0.03	0.015		
							CLI	0	0.25	0.06	0.06	0.06	0.03	0.015		
							GEM	5	16	1	1	2	0.25	0.5		S80F

^a CIP, ciprofloxacin; SPX, sparfloxacin; GAT, gatifloxacin; MOX, moxifloxacin; CLI, clinafloxacin; GEM, gemifloxacin.

^b Fold increase in MICs between the MIC for the quinolone-resistant strain selected in the quinolone-containing medium and the MIC for the parental strain. For example, the ciprofloxacin MIC for parent strain 1 was 0.25 μg/ml. After passages in ciprofloxacin containing medium, the ciprofloxacin MIC had increased to 32 μg/ml, i.e., a sevenfold increase.

^c Alterations refer to changes between the parent strain and the derived strain. All parent strains had wild-type GrlA and GyrA enzymes.

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