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Prevalence of *gyrA*, *gyrB*, *parC*, and *parE* Mutations in Clinical Isolates of *Streptococcus pneumoniae* with Decreased Susceptibilities to Different Fluoroquinolones and Originating from Worldwide Surveillance Studies during the 1997-1998 Respiratory Season

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From 8,419 worldwide clinical isolates of *Streptococcus pneumoniae* obtained during 1997-1998, 69 isolates with reduced susceptibility or resistance to fluoroquinolones (FQs) were molecularly characterized. For the isolates in this prevalence study, only *parC* (Ser-79→Tyr) and *gyrA* (Ser-81→Phe or Tyr) mutations, especially in combination, were found to contribute significantly to resistance. These mutations influenced the FQ MICs to varying degrees, although the rank order of activity remains independent of mutation type, with ciprofloxacin the least active, followed by levofloxacin, gatifloxacin/grepafloxacin/moxifloxacin/sparfloxacin/trovafloxacin, and clinafloxacin/sitafloxacin. Efflux likely plays a crucial role in reduced susceptibility for new hydrophilic FQs.

Streptococcus pneumoniae is a leading cause of illness in humans (32). Recent increases in resistance (4, 8, 9, 29–31) have spawned the development of several new fluoroquinolones (FQs) with improved in vitro antipneumococcal activity (1, 7, 10–12, 14, 15, 34, 35). In pneumococci, reports indicate mutations in *gyrA*, *gyrB*, *parC*, and *parE* to be associated with FQ resistance (16, 18, 20–23, 28, 33). Efflux is also reported to contribute significantly to reduced susceptibility for some hydrophilic FQs, such as ciprofloxacin, while more hydrophobic FQs, like grepafloxacin, appear less affected (5, 13).

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This work aimed to define the prevalence of predominant mutations conferring FQ resistance in pneumococci collected during 1 year. Mutations in genes conferring FQ resistance in *S. pneumoniae* (16, 18, 20–23, 28, 33) have been well studied, but studies have typically included either clinical isolates (few and locally derived) or laboratory-derived mutants. In contrast, this study, the largest molecular surveillance study of FQ resistance in *S. pneumoniae* to date, comprises clinically significant isolates from locations worldwide, providing the opportunity to characterize the prevalence of mutations globally and their impact on the MICs of several new FQs.

A total of 8,419 clinically significant isolates of *S. pneumoniae* associated with lower respiratory tract or blood infections were derived from 519 geographically distinct hospital laboratories in Austria, People's Republic of China, France, Germany, Italy, Japan, Spain, Switzerland, the United Kingdom, and the United States, in studies undertaken by MRL Pharmaceutical Services during 1997 and 1998 (24, 30; M. L. Hickey, C. Thornsberry, D. R. Diakun, S. V. Mani, and D. F. Sahm, Abstr. 38th Intersci. Conf. Antimicrob. Agents Chemother., abstr. E-20, 1998, and D. F. Sahm, I. A. Critchley, M. L. Hickey, D. R. Diakun, S. V. Mani, and C. Thornsberry, Clin. Micro. Infect., abstr. 110, 1999.) From these sources, 69 isolates were selected, including 30 isolates requiring MICs above the National Committee for Clinical Laboratory Standards susceptibility breakpoint (19) of any of the new FQs originally tested in the initial surveillance studies and 39 geographically unrelated isolates requiring MICs of levofloxacin ranging from 0.25 to 2 μg/ml. Together, these isolates provided a diverse strain set enabling the detection of mutations conferring high-level FQ resistance, as well as genetic changes reducing susceptibility. For each of the 69 isolates, MICs of each drug were determined in a single central laboratory by a broth microdilution assay according to the National Committee for Clinical Laboratory Standards (19). Each isolate was characterized with respect to mutations within *gyrA*, *gyrB*, *parC*, and *parE* with prepared chromosomal DNA (2) as templates for PCR amplification of target regions and with previously defined primers (16, 21) and methods (25).

The MIC distributions of each FQ tested are shown in Table 1. Overall, MICs of ciprofloxacin were highest, and MICs of sitafloxacin were lowest. For purposes of analysis, we considered sequence data in relation to MICs of levofloxacin to comprise the least active of the new FQs. Without exception, as is evident from Table 2, all of the 30 levofloxacin-resistant isolates (for which MICs were ≥4 μg/ml) had mutations within *gyrA* (alone or in combination with other mutations in *gyrA* or *parC*) encoding Ser-81→Phe or Tyr. No levofloxacin-susceptible isolates (for which MICs were ≤2 μg/ml) possessed these mutations. Of the levofloxacin-resistant isolates, 22 had mutations within *parC* (alone or in combination with other *parC* or *gyrA* mutations) encoding Ser-79→Phe, 3 had mutations encoding Asp-78→Asn, and 2 had mutations encoding Asp-

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TABLE 1. Number of isolates inhibited at different concentrations[a]

Antibiotic	MIC (μg/ml)										
	≤0.06	0.12	0.25	0.5	1	2	4	8	16	32	≥64
Ciprofloxacin			1	8	17	12	7	8	10	3	3
Levofloxacin			1	11	21	6	16	8	6		
Gatifloxacin	1	3	26	10	2	8	10	7	2		
Sparfloxacin	1	12	15	10	5	10	9	7			
Trovafloxacin	6	23	8	6	7	8	9	2			
Grepafloxacin	3	24	9	5	8	10	8	2			
Moxifloxacin	2	26	12	3	9	14	3				
Clinafloxacin	22	18	16	11	2						
Sitafloxacin	35	18	15	1							

[a] A total of 69 *S. pneumoniae* isolates were tested.

TABLE 2. Amino acid changes encoded by mutations in the *gyrA* and *parC* gene loci and corresponding fluoroquinolone MICs (mg/liter[a])

Mutant in locus		*n*	Antibiotic	No. of isolates for which corresponding MICs were										
gyrA	*parC*			≤0.06	012	0.25	0.5	1	2	4	8	16	32	≥64
—	—	29	Ciprofloxacin			1	6	14	7	1				
			Levofloxacin			1	6	17	5					
			Gatifloxacin	1	3	19	6							
			Sparfloxacin	1	10	9	7	2						
			Trovafloxacin	5	17	6	1							
			Grepafloxacin	3	17	7	2							
			Moxifloxacin	2	17	8	2							
			Clinafloxacin	18	11									
			Sitafloxacin	24	5									
—	Arg-95→Cys	2	Ciprofloxacin				1		1					
			Levofloxacin				1	1						
			Gatifloxacin				2							
			Sparfloxacin			1	1							
			Trovafloxacin		1	1								
			Grepafloxacin		1	1								
			Moxifloxacin		2									
			Clinafloxacin	1	1									
			Sitafloxacin	2										
—	Lys-137→Asn	6	Ciprofloxacin				1	3	2					
			Levofloxacin				4	2						
			Gatifloxacin			6								
			Sparfloxacin		2	4								
			Trovafloxacin	1	5									
			Grepafloxacin		6									
			Moxifloxacin		6									
			Clinafloxacin	3	3									
			Sitafloxacin	6										
—	Ser-79→Phe	2	Ciprofloxacin						1	1				
			Levofloxacin					1	1					
			Gatifloxacin				1	1						
			Sparfloxacin					1	1					
			Trovafloxacin				1	1						
			Grepafloxacin				1	1						
			Moxifloxacin			2								
			Clinafloxacin		2									
			Sitafloxacin	2										
Ser-81→Phe	—	2	Ciprofloxacin								1	1		
			Levofloxacin							1	1			
			Gatifloxacin						1	1				
			Sparfloxacin						1	1				
			Trovafloxacin						1	1				
			Grepafloxacin						1	1				
			Moxifloxacin					1	1					
			Clinafloxacin			1	1							
			Sitafloxacin			2								

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TABLE 2—*Continued*

Mutant in locus		*n*	Antibiotic	No. of isolates for which corresponding MICs were										
gyrA	*parC*			≤0.06	012	0.25	0.5	1	2	4	8	16	32	≥64
Ser-81→Tyr	—	3	Ciprofloxacin						1	1	1			
			Levofloxacin							3				
			Gatifloxacin				1	1	1					
			Sparfloxacin					2	1					
			Trovafloxacin			1	2							
			Grepafloxacin			1	2							
			Moxifloxacin		1	2								
			Clinafloxacin		1	2								
			Sitafloxacin	1	2	2								
Ser-81→Phe	Asp-78→Asn	1	Ciprofloxacin							1				
			Levofloxacin							1				
			Gatifloxacin						1					
			Sparfloxacin						1					
			Trovafloxacin					1						
			Grepafloxacin					1						
			Moxifloxacin					1						
			Clinafloxacin			1								
			Sitafloxacin		1									
Ser-81→Phe	Ser-79→Phe	16	Ciprofloxacin								4	6	3	3
			Levofloxacin							8	4	4		
			Gatifloxacin						3	8	5			
			Sparfloxacin					2	4	6	4			
			Trovafloxacin				1	3	6	6				
			Grepafloxacin					4	6	6				
			Moxifloxacin					5	9	2				
			Clinafloxacin			8	7	1						
			Sitafloxacin		8	8								
Ser-81→Phe	Ser-79→Phe	4	Ciprofloxacin								2	2		
	Lys-137→Asn		Levofloxacin								2	2		
			Gatifloxacin								2	2		
			Sparfloxacin							1	3			
			Trovafloxacin							2	2			
			Grepafloxacin						1	1	2			
			Moxifloxacin						3	1				
			Clinafloxacin			1	2	1						
			Sitafloxacin		2	1	1							
Ser-81→Phe	Lys-137→Asn	2	Ciprofloxacin							2				
	Asp-83→Asn		Levofloxacin							2				
			Gatifloxacin						2					
			Sparfloxacin						2					
			Trovafloxacin				1	1						
			Grepafloxacin					1	1					
			Moxifloxacin				1	1						
			Clinafloxacin			2								
			Sitafloxacin		1	1								
Ser-81→Phe	Asp-78→Asn	1	Ciprofloxacin							1				
	Arg-95→Cys		Levofloxacin							1				
			Gatifloxacin						1					
			Sparfloxacin						1					
			Trovafloxacin					1						
			Grepafloxacin					1						
			Moxifloxacin					1						
			Clinafloxacin			1								
			Sitafloxacin		1									
Ser-81→Phe	Asp-78→Asn	1	Ciprofloxacin									1		
	Lys-137→Asn		Levofloxacin								1			
			Gatifloxacin							1				
			Sparfloxacin							1				
			Trovafloxacin						1					
			Grepafloxacin						1					
			Moxifloxacin						1					
			Clinafloxacin				1							
			Sitafloxacin			1								

[a] A dash (—) denotes a wild-type sequence with no mutations identified. Position numbers are based on the *gyrA* (3) and *parC* (18, 20) sequence of *S. pneumoniae*.

83→Asn (Table 2). Twenty-eight single or combination mutations were found in *gyrB* and *parE* (including alterations Ala-639→Gln, Ala-538→Ser, Arg-541→Lys, Arg-545→Asn, Ala-639→Gln in GyrB; Glu-407→Lys, Lys-466→Met, Ile-460→Val, Asp-435→Asn, Ile-460→Val, and Pro-454→Ser in ParE). Compared to wild-type strains or strains with single or combinational mutations in *gyrA* or *parC* alone, with or without with these additional mutations in *gyrB* and *parE*, none was obviously associated with reduced susceptibility to any of the FQs, including ciprofloxacin or levofloxacin (although complementation studies would be necessary to confirm this as well as a comparative molecular analysis of fully susceptible isolates). Although some authors have described a possible role for a *parE* mutation in resistance (Asp-435→Asn) (17, 23) we and others have not been able to assign significance to *parE* (20) or *gyrB* (18) mutations.

The impact of well-characterized alterations in both laboratory mutants and clinical isolates, namely, Ser-81→Phe or Tyr in GyrA and Ser-79→Phe in ParC, previously described by other authors (16, 18, 20–22, 28, 33), was apparent (Table 2). Other alterations previously suggested as important, including Glu-85→Lys (laboratory mutant and clinical isolate) (21, 27, 33) or Trp-93→Arg (clinical isolate) (27) in GyrA and Ser-80→Pro (17) (clinical isolate) in ParC, were not found. While detected, alterations Arg-95→Cys (21) and Lys-137→Asn (27) in ParC seemed not to be significant. Thus, we conclude that such mutations are clinically rare or not obviously associated with FQ resistance. Asp-78→Asn and Asp-83→Asn alterations in ParC were only found in three and two isolates, respectively, and their contributions to FQ resistance were either negligible or masked, since they only occurred with a Ser-81→Phe alteration in GyrA. No previously unreported *parC*, *parE*, *gyrA*, or *gyrB* mutations significantly conferring reduced susceptibility to FQs were found. Thus only classical mutations, such as those in *parC* (Ser-79→Phe) and *gyrA* (Ser-81→Phe or Tyr), seem to play a significant role in FQ resistance in this worldwide sample of clinical *S. pneumoniae* isolates. Single significant mutations in *parC* or *gyrA* appeared to have moderate effects (approximately 2 dilution increases) on MICs, similar for each drug, although the high levels of activity of sitafloxacin and clinafloxacin reduced this effect.

These data underscore the probable impact of efflux on FQ susceptibility and the biovariation among strains observed when studying a diverse collection of clinical isolates in contrast to laboratory mutants. This is exemplified by the fact that many isolates with significant mutation(s) require MICs overlapping those for wild-type isolates (see Table 2). This overlap is most apparent with MICs required by isolates possessing single alterations of Arg-95→Cys or Lys-137→Asn in ParC, demonstrating the minimal impact of these mutations on susceptibility. It is especially noticeable when considering MICs of ciprofloxacin and sparfloxacin for isolates with multiple mutations in *parC* and *gyrA*. These quinolones comprise the most hydrophilic of the compounds tested and are readily effluxed; thus, higher MICs for isolates wild type at *gyrA* and *parC* loci can be observed. In contrast, hydrophobic compounds such as gatifloxacin, grepafloxacin, and moxifloxacin are less affected by efflux; thus, predictably, little or no MIC overlap occurs between isolates wild type at the topoisomerase and gyrase genes and those with detectable mutations in these loci. One-fold-dilution overlaps are observed for some mutational combinations for hydrophobic clinafloxacin and sitafloxacin, which can probably be explained by the extremely low MICs of these compounds and the reduced impact of mutational events on activity. These results are similar to data derived previously for efflux studies in *Staphylococcus aureus* (26).

The order of activity of drugs (Table 1 and 2) is generally conserved throughout, regardless of mutation(s) identified in *gyrA* and *parC*. Thus for all combinations of mutations detected, a left-to-right upward trend is evident (Table 2), with sitafloxacin as the most active compound and ciprofloxacin as the least active.

The results of this molecular epidemiological survey provide an opportunity to view the predominant mutations conferring reduced susceptibility to FQs in recent clinical pneumococcal isolates. Our findings indicate that researchers likely have characterized most of the mutations important in conferring reduced susceptibility to older FQ compounds, such as ciprofloxacin. Clearly, these mutations do impact susceptibilities to even the most active new FQs to some extent, although this varies between strains and for each drug. Based on the range of MICs of FQs for wild-type isolates, it is predicted that efflux will play a significant role for some drugs and warrants further study or that other systems have a hitherto-unidentified impact on FQ susceptibility. It will be interesting to witness the effect of selective pressures imposed on these genetic systems by the increased use of the new FQ compounds described in this study, many of which retain high levels of in vitro activity despite the presence of significant mutations in topoisomerase- and gyrase-encoding genes. This is particularly significant in light of recent work by Chen et al. (6), who report an increasing prevalence of pneumococcal resistance to fluoroquinolones. Future prevalence studies will be able to track changes in the predominant mutations conferring resistance to FQs.

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