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Efflux and target mutations as quinolone resistance mechanisms in clinical isolates of *Streptococcus pneumoniae*

John Broskey, Ken Coleman*, Mick N. Gwynn, Lynn McCloskey, Chris Traini, LeRoy Voelker and Richard Warren

SmithKline Beecham Pharmaceuticals, Collegeville, PA, USA

The aim of this study was to characterize quinolone resistance mechanisms in strains of *Streptococcus pneumoniae* with increased MICs of ofloxacin. These strains were also tested for their susceptibility to a battery of quinolone antimicrobial agents, including gemifloxacin. Of the *S. pneumoniae* isolates used, 27 were susceptible to ofloxacin, 18 intermediate and 48 resistant (ofloxacin MIC <4, 4 and >4 mg/L, respectively). In general, the ofloxacin-susceptible strains had no amino acid substitutions in GyrA, GyrB, ParC or ParE. Moderate increases in MIC were associated with substitutions in the quinolone resistance-determining region (QRDR) of ParC, while the highest MICs were found for strains that also had substitutions in the QRDR of GyrA. The most common substitutions were Ser79→Phe in ParC and Ser81→Phe in GyrA. Other substitutions were identified within the QRDR of ParC and outside the QRDR of ParC and ParE; these did not appear to affect susceptibility. The effects of antimicrobial efflux pumps were studied by determining MICs of a range of quinolones in the presence and absence of reserpine, an inhibitor of Gram-positive efflux pumps. Our results indicated that high-level resistance, caused entirely by efflux, was seen in a minority of ofloxacin-resistant *S. pneumoniae* strains. Testing the susceptibility of quinolone-resistant strains to gemifloxacin, ciprofloxacin, norfloxacin, ofloxacin and trovafloxacin revealed that gemifloxacin was least affected by this large variety of resistance mechanisms and was the only quinolone with MICs of ≤0.5 mg/L for all strains in this study. These results suggest that gemifloxacin is highly potent against *S. pneumoniae* and may also be effective against strains resistant to other quinolones.

Introduction

Resistance to currently available quinolone antimicrobial agents among *Streptococcus pneumoniae* is an increasing problem worldwide.[1,2] It is thought that the targets of the quinolones are the type II topoisomerases DNA gyrase and DNA topoisomerase IV,[3,4] which are responsible for topological transformations of DNA. Quinolone resistance is frequently associated with mutations of these targets, although drug efflux is another mechanism of resistance in certain organisms, including *S. pneumoniae*.[5–7] The novel quinolone gemifloxacin is active against streptococci that are resistant to other quinolones.[1,8,9]

In order to determine the factors contributing to quinolone resistance in pneumococci, we studied 93 strains of *S. pneumoniae* with varying levels of ofloxacin resistance. The quinolone resistance-determining regions (QRDRs) of the *gyrA*, *gyrB*, *parC* and *parE* genes were sequenced to determine any mutations in these regions which could alter the encoded topoisomerase subunits. The role of efflux pumps in these resistant strains was also studied by determining the MICs of a range of quinolones in the presence and absence of reserpine, an inhibitor of Gram-positive efflux pumps.

Materials and methods

Compounds

Gemifloxacin (batch no. 03R1P2-1-1, potency 73.8%) was obtained from SmithKline Beecham, Harlow, UK. Trovafloxacin (batch no. 2538-086-02, free base, purity 81.5%)

*Correspondence address. Microbiology Research, SmithKline Beecham Pharmaceuticals, 1250 S. Collegeville Road, Collegeville, PA 19426-0989, USA. Tel: +1-610-917-6273; Fax: +1-610-917-4617; E-mail: Kenneth_Coleman@SBPHRD.com

was obtained from Pfizer Pharmaceuticals, Groton, CT, USA. Ciprofloxacin was deformulated by SmithKline Beecham Pharmaceuticals, and reserpine and ofloxacin were obtained from Sigma Chemical Co., St Louis, MO, USA.

Isolates

S. pneumoniae isolates with ofloxacin MICs of ⩾4 mg/L were obtained from the 1992–1998 Alexander Project,[10] the 1996 SENTRY[11] and SPAR[12] surveillance studies, the 1997 ALERT[13] surveillance project and the University of Iowa (Iowa City, IA, USA) and SB Clinical Laboratories (West Norriton, PA, USA). *S. pneumoniae* isolates with ofloxacin MICs of <4 mg/L were selected at random from the 1998 Alexander Project.

Media

MICs were determined using the NCCLS method for broth microdilution[14] with cation-adjusted Mueller–Hinton broth (Becton Dickinson, Cockeysville, MD, USA) with 5% lysed horse blood (Becton Dickinson). Reserpine inhibition studies were carried out in Todd–Hewitt broth supplemented with 5% yeast extract (Becton Dickinson). Reserpine 80 mg/L was added to inocula to inhibit proton motive force efflux pump activity.

Microdilution

MICs were determined by broth microdilution in accordance with NCCLS recommended procedures.[14] Where appropriate, reserpine 80 mg/L was added to the medium to inhibit efflux pump activity. All MIC determinations were carried out in triplicate.

Doubling dilution series were prepared for test compounds in 96-well microtitre plates. The dilution ranges used were 2–0.002 mg/L for gemifloxacin and 32–0.03 mg/L for trovafloxacin, ciprofloxacin and ofloxacin. A positive growth control well, containing medium and test isolate, was included on each plate. A 10 μL sample of test medium from the positive growth control well was plated to determine the purity of the test isolate.

Isolation of S. pneumoniae chromosomal DNA

Chromosomal DNA was prepared by resuspending a single plated colony in 1 mL sterile water. Cells were then pelleted and the supernatant removed. Pellets were mixed with 200 μL chelating matrix (6% Bio-Rad InstaGene Matrix; Bio-Rad Labs, Hercules, CA, USA) incubated at 56°C for 30 min, mixed by vortex and then lysed in a 100°C water bath for 8 min. The mixture was mixed by vortex again, centrifuged and then added directly to the PCR reaction (20 μL supernatant per 50 μL PCR reaction). Each 50 μL PCR reaction contained 200 ng of DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 100 pmol of each primer and 2.5 U of *Taq* DNA polymerase (Life Technologies, Rockville, MD, USA). The target gene was amplified by 35 cycles at 95°C for 45 s, 53°C for 30 s and 72°C for 90 s. A final incubation step with an additional 10 min at 72°C was used. Amplified products were checked by agarose gel electrophoresis, then purified using Qiagen QIA quick spin columns (Quiagen Inc., Valencia, CA, USA). The primers used to amplify the *gyrA*, *gyrB*, *parC* and *parE* genes were as follows:

gyrA_pcr1, 5′-TTAAAAAACTTTGTCACGAATATGCC-3′;
gyrA_pcr2, 5′-AACGATACGCTCACGACCAGT-3′;
gyrB_pcr1, 5′-TGAAGGACAAACCAAGACCAAA-3′;
gyrB_pcr2, 5′-GTCCATTTCACCTAGCCCCTTATA-3′;
parC_pcr1, 5′-AAAACCTACTCTACATTCTTTGAAAGGAG-3′;
parC_pcr2, 5′-CAGTTGGGTGGTCAATCATGTAAA-3′;
parE_pcr1, 5′-AGGTTCAGACTATCGTGAGGGACTAGC-3′;
parE_pcr2, 5′-CCGACTCTAATTTCCAGTTTACTAAC-3′.

DNA sequencing

PCR products were prepared for automated DNA sequencing using an ABI Prism 377 DNA sequencer (Perkin–Elmer ABI, Foster City, CA, USA). Cycle sequencing reactions were carried out with 50 ng DNA and 3.2 pmol primer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer ABI). The oligonucleotides used to prime these DNA sequencing reactions were as follows:

gyrA_F1, 5′-CGTTTTAGTGGTTTAGAGGC-3′;
gyrA_R1, 5′-GACCAACTTCACTGCATCA-3′;
gyrB_F1, 5′-TTCTCCGATTTCCTCATG-3′;
gyrB_R1, 5′-CCCGGCTGGATATATTCT-3′;
parC_F1, 5′-CGCCCTAGATACTGTGTGA-3′;
parC_R1, 5′-AAATCCCAGTCGAACCAT-3′;
parE_F1, 5′-TGTGGATGGAATAGTGGC-3′;
parE_R1, 5′-ACCGAACTGTTTACGGAGT-3′.

The DNA sequences were assembled and edited with Sequencher V3.0 (Gene Codes Corp., Ann Arbor, MI, USA).

Results

Of the 93 isolates reported here, the 12 most susceptible had ofloxacin MICs of 0.125–0.5 mg/L (Table I). In general, these 12 isolates showed no amino acid changes within the QRDRs of GyrA, GyrB, ParC or ParE, although two isolates did show a Ser114→Gly substitution in GyrA and the double substitution of Ser52→Gly + Asn91→Asp in ParC. These same substitutions were also found in three strains with MICs of 1–4 mg/L, but as they appeared to have no effect on the MICs for susceptible isolates they are assumed to be silent. Similarly, a number of seemingly

Table I. Number of isolates showing various target substitutions by ofloxacin MIC

Ofloxacin MIC (mg/L)	*n*	Strains (*n*)											
				ParC					GyrA				
		Efflux[a]	wt	Ser79→Phe	Ser79→Tyr	Asp83→Asn	Asp83→Tyr	Arg95→Cys	wt	Ser81→Phe	Ser81→Tyr	Glu85→Lys
⩽0.5	12	4	12						12			
1	13	11	12		1				13			
2	2	1	1				1		2			
4	18	7	5	4	6	3			18			
8	3	1		2		1			3			
16	11	4		7	1	2		1	1	10		
32	30	9		23	6	1			1	22	3	4
64	3	2		1	2						1	2
>64	1	0		1							1	

wt, wild type.
[a]Almost all strains showed a one-dilution increase in MIC.

silent substitutions were found outside the QRDR region of ParE in strains of all susceptibilities. The most common of these was Ile460→Val, but His351→Leu, His347→Leu and Cys329→Ser were also found, usually together. Ofloxacin is not pumped out by efflux to any great extent, but four of these 12 strains (33.3%) showed a reserpine-inhibitable efflux mechanism which generally doubled the ofloxacin MIC.

Only two of the 15 strains with an ofloxacin MIC of 1–2 mg/L had a substitution in ParC (one Ser79→Tyr and one Asp83→Tyr), and all had wild-type GyrA, GyrB and ParE. This group was notable for the high incidence of efflux observed. Ofloxacin efflux was observed in about one-third of the population overall, but in this MIC group 87% of strains (13/15) showed an efflux mechanism which increased the ofloxacin MIC from 0.5 to 1 mg/L.

In the 21 strains with ofloxacin MICs of 4–8 mg/L, no substitutions were found in GyrA or GyrB and only one showed a change in ParE. Most showed a single amino acid substitution in the QRDR of ParC, either Ser79→Phe, Ser79→Tyr or Asp83→Asn. For the 20 single-step mutants included in this study, 13 were found at the modal MIC of 4 mg/L (range 1–32 mg/L). One strain with wild-type GyrA and ParC did have an Asp435→Asn substitution in ParE which may account for the slightly higher MIC for this strain (4 mg/L). One isolate had the double ParC substitution of Ser52→Gly + Asn91→Asp, assumed to have no impact on the MIC (see above), but this and two other isolates had no detectable substitutions that might explain the ofloxacin MICs of 4 mg/L.

Of the 45 strains with ofloxacin MICs of ⩾16 mg/L, all but two had single substitutions in both GyrA and ParC. The most common GyrA substitution was Ser81→Phe (32/45 isolates), but five strains had a Ser81→Tyr substitution and six a Glu85→Lys substitution. The most common ParC substitution was Ser79→Phe (32/45 strains), but Ser79→Tyr, Asp83→Asn and Arg95→Cys substitutions were also seen (in nine, three and one of the 45 strains, respectively). The highest MICs were for the two strains with GyrA and ParC substitutions of Ser81→Tyr and Ser79→Phe, respectively.

Two isolates with ofloxacin MICs of 32 and 16 mg/L had no substitutions in GyrA and a single substitution in ParC (Ser79→Tyr). No significant efflux of ofloxacin was seen for either of these isolates, though their efflux of ciprofloxacin was higher.

In general, amino acid substitutions within the QRDRs of ParC and GyrA have much the same effect on the MICs of gemifloxacin, trovafloxacin and ciprofloxacin as they have on ofloxacin. The major differences seen between antimicrobial agents are the range of MICs and degree of efflux activity. Ciprofloxacin (0.25–64 mg/L) and ofloxacin (0.125–64 mg/L) have the highest MIC ranges, with trovafloxacin MICs being lower (0.008–16 mg/L) and gemifloxacin (MICs 0.004–0.5 mg/L) being the only quinolone with MICs of <1 mg/L for all the isolates tested.

Efflux of trovafloxacin and ofloxacin in this study was low. Only 40% of isolates showed efflux, and in most cases the result was a doubling of the MIC. Gemifloxacin efflux occurred in 55% of all isolates (Table II), with two-thirds of this group showing a doubling of MIC and the remainder a four- to eight-fold increase. Ciprofloxacin efflux occurred in almost all isolates, including the most susceptible (Table III), with most isolates showing at least a four-fold increase in MIC attributable to efflux. The four isolates with the greatest ciprofloxacin efflux were, interestingly, the four isolates with ofloxacin MICs of 4 mg/L and no discernible target mutations.

The susceptibility of these isolates to trovafloxacin (≤0.016–16 mg/L) was also determined. Trovafloxacin was more potent than ciprofloxacin and ofloxacin but was generally about 8- to 16-fold less active than gemifloxacin. Trovafloxacin efflux was low, like that of ofloxacin.

Discussion

There have been a number of reports of a two-step mechanism of acquisition of quinolone resistance in *S. pneumoniae*; the first step is associated with an amino acid substitution in ParC and the second with an additional substitution in GyrA,[6,15–17] although there is evidence that some quinolones select for a first mutation in *gyrA*.[18,19] In this study, all clinical isolates showed a first-step mutation in the QRDR of ParC, with a higher level of resistance arising after a second mutation in the QRDR of *gyrA*.This does not imply that the primary mutational site for gemifloxacin is *parC*. In fact, it is likely to be *gyrA*.[20] The most effective, and most common, substitutions appear to be Ser79→Phe in ParC and Ser81→Tyr in GyrA, and the two most resistant isolates in this study had both of these substitutions.

Quinolone resistance in *S. pneumoniae* as a result of drug efflux has been reported previously[5,21,22] and the sequence of one pump, PmrA, has recently been reported.[23] In this study, almost all isolates, including most of the highly susceptible strains, showed some evidence of ciprofloxacin efflux. Most isolates (41%) showed a doubling in MIC, but 33% demonstrated a four-fold increase and 19% ≥8-fold increase. In only five isolates was the elevated MIC entirely attributable to efflux, and all five had an intermediate susceptibility to ofloxacin (MIC 4 mg/L), with ciprofloxacin MICs of 4–8 mg/L. Ofloxacin and trovafloxacin efflux occurred in about one-third of the isolates in each susceptibility range reported above, but in the group with ofloxacin MICs of 1–2 mg/L, ofloxacin efflux occurred in 80% of isolates. Pan *et al.*[4] have reported a first-step resistance level which they attribute entirely to reduced permeability, and our results would seem to confirm their findings. The clinical significance of efflux-mediated quinolone resistance in *S. pneumoniae* is unclear, however.

Susceptibility testing of quinolone-resistant strains to gemifloxacin and other test quinolones revealed that gemifloxacin was the agent least affected by the various resistance mechanisms. Gemifloxacin was the most potent agent tested, and was the only quinolone with MICs of ≤0.5 mg/L for all strains (MIC range 0.004–0.5 mg/L). These results indicate that gemifloxacin is highly potent against *S. pneumoniae*, and may also be effective against strains resistant to other quinolones.

Table II. Number of isolates showing gemifloxacin efflux

Gemifloxacin MIC (mg/L)	*n*	Fold increase in MIC			
		0	2	4	8
≤0.008	14	3	8	3	
0.015	11	1	6	3	1
0.03	16	9	4	1	2
0.06	8	6	0	1	1
0.13	11	6	3	1	1
0.25	29	14	11	4	
0.5	4	1	3		

Table III. Number of isolates showing ciprofloxacin efflux

Ciprofloxacin MIC (mg/L)	*n*	Fold increase in MIC				
		0	2	4	8	>8
≤0.5	17		9	6	2	
1	4			3	1	
2	8		2	2	2	2
4	14	3	5	2	4	
8	7	1	3		1	2
16	7	1	2	3		1
32	23	1	16	5		1
64	12		1	9	2	
>64	1			1		

References

1. Chen, D. K., McGeer, A., de Azavedo, J. C. & Low, D. E. (1999). Decreased susceptibility of *Streptococcus pneumoniae* to fluoroquinolones in Canada. *New England Journal of Medicine* **341**, 233–9.

2. Felmingham, D. & Washington, J. (1999). Trends in the antimicrobial susceptibility of bacterial respiratory tract pathogens—findings of the Alexander Project 1992–1996. The Alexander Project Group. *Journal of Chemotherapy* **11**, *Suppl. 1*, 5–21.

3. Tankovic, J., Perichon, B., Duval, J. & Courvalin, P. (1996). Contribution of mutations in *gyrA* and *parC* genes to fluoroquinolone resistance of mutants of *Streptococcus pneumoniae* obtained *in vivo* and *in vitro*. *Antimicrobial Agents and Chemotherapy* **40**, 2505–10.

4. Pan, X.-S., Ambler, J., Mehtar, S. & Fisher, L. M. (1996). Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **40**, 2321–6.

5. Baranova, N. N. & Neyfakh, A. A. (1997). Apparent involvement of a multidrug transporter in the fluoroquinolone resistance of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **41**, 1396–8.

6. Munoz, R. & De La Campa, A. G. (1996). ParC subunit of DNA topoisomerase IV of *Streptococcus pneumoniae* is a primary target of fluoroquinolones and co-operates with DNA gyrase A subunit in forming resistance phenotype. *Antimicrobial Agents and Chemotherapy* **40**, 2252–7.

7. Zeller, V., Janoir, C., Kitzis, M. D., Gutmann, L. & Moreau, N. J. (1997). Active efflux as a mechanism of resistance to ciprofloxacin in *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **41**, 1973–87.

8. Heaton, V. J., Goldsmith, C., Ambler, J. & Fisher, L. M. (1999). Gemifloxacin is highly active against both penicillin-resistant and ciprofloxacin-resistant *Streptococcus pneumoniae*. *Journal of Antimicrobial Chemotherapy* **44**, *Suppl. A*, 140 (Abstract P452).

9. Brenwald, N., Gill, M., Boswell, F. J. & Wise, R. (1999). Activity of gemifloxacin against *Streptococcus pneumoniae* strains resistant to fluoroquinolones by an efflux mechanism. *Journal of Antimicrobial Chemotherapy* **44**, *Suppl. A*, 145 (Abstract P477).

10. Schito, G. C. (1996). Alexander Project Symposium. Introduction. *Diagnostic Microbiology and Infectious Disease* **25**, 151–224.

11. Pfaller, M. A., Jones, R. N., Doern, G. V., Kugler, K. & the SENTRY Participants Group. (1998). Bacterial pathogens isolated from patients with bloodstream infection: frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY antimicrobial surveillance program (United States and Canada, 1997). *Antimicrobial Agents and Chemotherapy* **42**, 1762–70.

12. Ballow, C. H., Jones, R. N., Johnson, D. M., Deinhart, J. A. & Schentag, J. J. (1997). Comparative *in vitro* assessment of sparfloxacin activity and spectrum using results from over 14,000 pathogens isolated at 190 medical centers in the USA. SPAR Study Group. *Diagnostic Microbiology and Infectious Disease* **29**, 173–86.

13. Mortensen, J. E., Jacobs, M. R. & Koeth, L. M. (1998). Susceptibility of *Streptococcus pneumoniae* and *Haemophilus influenzae* in 1997: Results of a hospital-based study. In *Program and Abstracts of the Thirty-Eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA, 1998*. Abstract C-19, p. 74. American Society for Microbiology, Washington, DC

14. National Committee for Clinical Laboratory Standards. (1997). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Fourth Edition: Approved Standard M7-A4*. NCCLS, Wayne, PA.

15. Janoir, C., Zeller, V., Kitzis, M. D., Moreau, N. J. & Gutmann, L. (1996). High level fluoroquinolone resistance in *Streptococcus pneumoniae* requires mutations in *parC* and *gyrA*. *Antimicrobial Agents and Chemotherapy* **40**, 2760–4.

16. De La Campa, A. G., Garcia, E., Fenoll, A. & Munoz, R. (1997). Molecular bases of three characteristic phenotypes of pneumococcus: optochin-sensitivity, coumarin-sensitivity and quinolone-resistance. *Microbial Drug Resistance* **3**, 177–93.

17. Jorgensen, J. H., Weigel, L. M., Ferraro, M. J., Swenson, J. M. & Tenover, F. C. (1999). Activities of newer fluoroquinolones against *Streptococcus pneumoniae* clinical isolates including those with mutations in the *gyrA*, *parC* and *parE* loci. *Antimicrobial Agents and Chemotherapy* **43**, 329–34.

18. Pan, X.-S. & Fisher, L. M. (1997). Targeting DNA gyrase in *Streptococcus pneumoniae* by sparfloxacin: selective targeting of gyrase or topoisomerase IV by quinolones. *Antimicrobial Agents and Chemotherapy* **41**, 471–4.

19. Varon, E., Janoir, C., Kitzis, M. D. & Gutmann, L. (1999). ParC and GyrA may be interchangeable initial targets of some fluoroquinolones in *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **43**, 302–6.

20. Heaton, V. J., Ambler, J. & Fisher, L. M. (1999). Genetic analysis of gemifloxacin action in *Streptococcus pneumoniae.* In *Program and Abstracts of the Thirty-Ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, USA, 1999*. Abstract 1394, p. 125. American Society for Microbiology, Washington, DC.

21. Brenwald, N. P., Gill, M. J. & Wise, R. (1997). The effect of reserpine, an inhibitor of multi-drug efflux pumps, on the in-vitro susceptibilities of fluoroquinolone-resistant strains of *Streptococcus pneumoniae* to norfloxacin. *Journal of Antimicrobial Chemotherapy* **40**, 458–60.

22. Brenwald, N. P., Gill, M. J. & Wise, R. (1998). Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **42**, 2032–5.

23. Gill, M. J., Brenwald, N. P. & Wise, R. (1999). Identification of an efflux pump gene, *pmrA*, associated with fluoroquinolone resistance in *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* **43**, 187–9.