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Molecular Characterization of Rarely Isolated Garenoxacin-Resistant *Streptococcus pneumoniae* Collected from the SENTRY Antimicrobial Surveillance Program (1999-2005)

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Amended Abstract

Background: Although fluoroquinolone (FQ) resistance (R) rates among *S. pneumoniae* (SPN) remains very limited, increasing selective pressure due to escalating use and clonal dissemination may lead to greater R rates in the future. This study was performed to determine which quinolone resistance determining region (QRDR) mutations were required to produce elevated SPN MIC values including high-level R (≥4 µg/ml) to garenoxacin (GRN), a potent novel des-F(6) quinolone.

Methods: During 1999-2005, a total of 18,402 SPN were collected from North America, Latin America and Europe and tested for susceptibility (S) to various quinolones using reference broth microdilution methods (CLSI, M7-A7 and M100-S16, 2006). A subset of these isolates with GRN MIC values of ≤0.06->4 µg/ml were tested for QRDR mutations in *gyrA* or *B* and *parC* or *E* by PCR and sequencing.

Results: Among the SPN strains tested, $MIC_{50/90}$ values for GRN were 0.06 µg/ml and only 0.4% had GRN MIC values >1 µg/ml (0.02% at >4 µg/ml). The QRDR mutations associated with an elevated GRN MIC (≥0.12 µg/ml) are shown in the table (wild-type GRN MIC values at ≤0.06 µg/ml):

		No. of mutations	
GRN MIC (µg/ml)	No. of strains	Topoisomerase IV (*parC/E*)	Topoisomerase IV + DNA Gyrase (*gyrA/B* and *parC/E*)
0.12	53	40	11
0.25	11	2	9
0.5	34	4	29
1	35	1	34
≥2	8[a]	0	8[b]

a. Included GRN MIC values of 2 µg/ml (three strains), 4 µg/ml (two strains) and >32 µg/ml (three strains).
b. Mutations required for high-level R (GRN MIC, ≥4 µg/ml) included *parC* S79F and D83Y, and *gyrA* S81F or Y and E85K.

Conclusions: Over 75% of isolates with a slightly elevated GRN MIC value (0.12 µg/ml) had mutations only in topoisomerase IV, usually in *parE*. In contrast, all but one isolate tested with GRN MIC values at ≥1 µg/ml required multiple mutations in both the topoisomerase IV and DNA gyrase genes and expressed high-level R to LEVO (MIC, >32 µg/ml). It is important to continually monitor the susceptibility of newer and investigational quinolones as this class continues to be used to treat respiratory tract infections among other indications.

Introduction

Currently, the resistance rate among *Streptococcus pneumoniae* to the fluoroquinolone class is fairly low. However, there are many reports documenting an underlying population of strains possessing first-step *parC* gene mutations located within the topoisomerase IV of the quinolone resistance-determining region (QRDR). This subpopulation of strains are likely to be responsible for clinical failure upon fluoroquinolone therapy due to the higher probability of acquiring additional mutations in DNA gyrase (*gyrA* and *gyrB*) which typically leads to higher-level resistance to many of the fluoroquinolones currently used in clinical practice. Some studies have demonstrated a slight increase in fluoroquinolone resistance over time which suggests that the prevalence of QRDR mutations may be increasing. However, the potencies of the fluoroquinolones have increased during the development of "newer generation" compounds when tested against *S. pneumoniae* and other Gram-positive pathogens.

Garenoxacin is a des-F(6) quinolone with an excellent potency against isolates associated with pneumonia. The purpose of this study was to compare the potency of garenoxacin to other fluoroquinolones against *S. pneumoniae* collected from a large surveillance network during 1999-2005. A subset of isolates with garenoxacin MIC values that ranged from the modal value to high-level resistance was evaluated for QRDR mutations and the effect shown upon several other fluoroquinolones.

Materials and Methods

During a seven-year period, a total of 18,402 isolates of *S. pneumoniae* were tested against garenoxacin, of which, 8,869 had MIC comparisons against five other fluoroquinolones and are presented in this study (Table 1). Among these isolates, 232 strains were included in the investigation of QRDR mutation analysis. These isolates were collected from 67 medical centers in the United States (36 centers), Canada (five centers), Europe (11 countries; 20 centers) and Latin America (three countries; six centers) which were participants in the SENTRY Antimicrobial Surveillance Program during 1999-2005. This subset collection of clinical isolates were selected to include varying MIC values to garenoxacin (Table 2).

The isolates were tested for susceptibility to ciprofloxacin, levofloxacin, garenoxacin, gatifloxacin, gemifloxacin and moxifloxacin using CLSI broth microdilution methods (M7-A7, 2006) and Etest using manufacturer's recommendations (AB BIODISK, Solna, Sweden). Strains were identified by local sites and forwarded to the reference laboratory (JMI Laboratories, North Liberty, IA or University of Iowa Healthcare, Iowa City, IA) which confirmed identification by bile solubility and the optochin susceptibility tests. Susceptibility criteria were those defined by the Clinical and Laboratory Standards Institute (CLSI) recommendations (M100-S16, 2006).

Topoisomerase IV and gyrase A gene segments responsible for fluoroquinolone resistant phenotype were amplified as described earlier. The primers used were as follows: parC-F: 5′ TGA CAA GAG CTA CCG TAA GTC G 3′, parC-R: 5′ TCG AAC CAT TGA CCA AGA GG 3′, parE-F: 5′ ACG TAA GGC GCG TGA TGA G 3′, parE-R: 5′ CTA GCG GAC GCA TGT AAC G 3′, gyrA-F: 5′ CGT CGC ATT CTC TAC GGA 3′, gyrA-R: 5′ TCT TGC TCA TAC GTG CCT CGG 3′. PCR products were cleaned using QIAquick PCR purification kit (QIAGEN GmbH, Germany). The cleaned QRDR amplicons were sequenced using Sanger-based dideoxy sequencing strategy involving the incorporation of fluorescent dye-labeled terminators into the sequencing reaction products. Sequences obtained were subjected to NCBI BLAST search to determine mutations present in the QRDR region of the fluoroquinolone resistant isolates. QRDR sequences of *S. pneumoniae* S6, a well characterized fluoroquinolone susceptible isolate were used as a control.

Results

- Garenoxacin had excellent potency against *S. pneumoniae* with a MIC_{50} and MIC_{90} value of 0.06 µg/ml (Table 1). This was superior to all other fluoroquinolones (MIC_{90} values, 0.25-2 µg/ml) except for gemifloxacin (MIC_{90}, 0.03 µg/ml).

Table 1. Distribution of MIC values for six fluoroquinolones tested against 8,869 strains of *S. pneumoniae* isolates collected from medical centers during the 1999-2005 SENTRY Program.

	MIC (µg/ml)		
Antimicrobial agent	50%	90%	Range
Ciprofloxacin	1	2	≤0.03->4
Levofloxacin	1	1	≤0.5->4
Garenoxacin	0.06	0.06	≤0.03->4
Gatifloxacin	0.25	0.5	≤0.5->4
Gemifloxacin	0.015	0.03	≤0.008->1
Moxifloxacin	0.12	0.25	≤0.03->4

Table 2. Location of mutations of the QRDR associated with variable levels of garenoxacin MIC elevations.

		No. of mutations (%)		
Garenoxacin MIC (µg/ml)	No. of strains	Topoisomerase IV only (*parC/E*)	Topoisomerase IV + DNA Gyrase (*gyrA/B* and *parC/E*)	None
≤0.06	91[a]	63 (69.2)	9 (9.9)	19 (20.9)
0.12	53	40 (75.5)	11 (20.8)	2 (3.8)
0.25	11	2 (18.2)	9 (81.8)	0 (0.0)
0.5	34	4 (11.8)	29 (85.3)	1 (2.9)
1	35	1(2.9)	34 (97.1)	0 (0.0)
≥2	8[b]	0 (0.0)	8 (100.0)	0 (0.0)

a. Included four strains with MIC values ≤0.03 µg/ml and 87 strains with an MIC of 0.06 µg/ml.
b. Included strains with the following MIC values: 2 µg/ml (3 strains), 4 µg/ml (2 strains) and >32 µg/ml (2 strains).

- Approximately 10% of isolates with a garenoxacin MIC value of ≤0.06 µg/ml had mutations in both topoisomerase IV and DNA gyrase (Table 2). This rate doubled among strains with a MIC 0.12 µg/ml and significantly increased with strains at a MIC of 0.25-0.5 µg/ml (81.8-85.3%). Nearly all of strains (97%) with a garenoxacin MIC of ≥1 µg/ml had multiple QRDR mutations.
- Table 3 describes the QRDR mutations that have been described in the literature as being responsible for elevated fluoroquinolone MIC values. The most common amino acid substitutions were located at positions serine 81 (*gyrA*) and serine 79 (*parC*). Only four isolates had *gyrB* mutations and had only slightly elevated garenoxacin MIC values (≤0.12 µg/ml). Nearly 60% of the isolates had the relatively "silent" *parE* mutation (valine substitution for isoleucine at position 460).
- Among the *S. pneumoniae* isolates with garenoxacin MIC values of 0.06 µg/ml, 42.5% of strains were shown to have *parC* mutations and typically only had modestly elevated MIC values to levofloxacin (1-2 µg/ml) and ciprofloxacin (2-4 µg/ml).
- Three *S. pneumoniae* isolates had garenoxacin MIC values of >32 µg/ml. These isolates had one or two QRDR mutations in both *gyrA* and *parC*, and were highly resistant to all tested fluoroquinolones.

Table 3. The QRDR amino acid positions where mutations were detected among *S. pneumoniae* isolates with garenoxacin MIC values ranging from ≤0.06 to >32 µg/ml.

	Numbers (%) of QRDR mutations									
	gyrA[a]		*gyrB*[b]	*parC*[c]					*parE*[d]	
Garenoxacin MIC (µg/ml)	S81	E85	D435	S79	K137	D91	D83	S52	I450	D435
≤0.06	3 (3.3)	1 (1.1)	3 (3.3)	14 (15.4)	22 (24.2)	2 (2.2)	4 (4.4)	3 (3.3)	51 (56.0)	1 (1.1)
0.12	2 (3.8)	1 (1.9)	1 (1.9)	17 (32.1)	7 (13.2)	8 (15.1)	2 (3.8)	2 (3.8)	41 (77.4)	2 (3.8)
0.25	9 (81.8)	-[e]	-	4 (36.4)	-	2 (18.2)	2 (18.2)	-	6 (54.6)	1 (9.1)
0.5	29 (85.3)	-	-	23 (67.7)	7 (20.6)	5 (14.7)	3 (8.8)	1 (2.9)	14 (41.2)	3 (8.8)
1	29 (82.9)	5 (14.3)	-	33 (94.3)	12 (35.3)	10 (28.6)	3 (8.6)	2 (5.7)	20 (57.0)	-
2	-	3 (100.0)	-	3 (100.0)	1 (33.3)	1 (33.3)	-	-	2 (66.7)	-
4	1 (50.0)	1 (50.0)	-	2 (100.0)	-	-	-	-	1 (50.0)	-
>32	3 (100.0)	2 (66.7)	-	3 (100.0)	-	-	1 (33.3)	-	-	-

a. Other mutations noted at amino acid positions A17, V71 and S114.
b. One other mutation noted at amino acid position K469.
c. Other mutations noted at amino acid positions G77, Y129, E135 and A142.
d. Other mutations noted at amino acid positions N377, I493 and E494.
e. - = no mutations were noted at this position.

Conclusions

- A QRDR mutation in *gyrA* usually at amino acid position S81 is typically required to elevate the garenoxacin MIC to >0.12 µg/ml. This is most often associated with a *parC* serine substitution at amino acid position 79.
- There is a variable effect that QRDR mutations have on the MIC of different fluoroquinolones and *S. pneumoniae*. Strains that are high-level resistant to levofloxacin and ciprofloxacin (MIC, >32 µg/ml) can still be inhibited by garenoxacin concentrations of 0.12-4 µg/ml.
- A recent report (Zhanel et al., 2006) has shown that garenoxacin was bactericidal against *S. pneumoniae* that are susceptible to ciprofloxacin, as well as those strains resistant due to *parC* mutations and/or efflux mechanisms. This finding was also noted by the SENTRY Program for levofloxacin-resistant strains (Anderegg et al., 2004).

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