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Prevalence of First-Step Mutants among Levofloxacin-Susceptible Invasive Isolates of *Streptococcus pneumoniae* in the United States

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By use of a PCR-restriction fragment length polymorphism assay, we screened 496 levofloxacin-susceptible invasive pneumococcal strains (MIC ≤ 2 mg/liter) for quinolone resistance-determining region mutations known to confer fluoroquinolone resistance. Among those with a levofloxacin MIC of 2 mg/liter, 16.2% of isolates recovered from nursing home residents and 6.4% from non-nursing home residents had first-step mutations.

The acquisition of mutations in the quinolone resistance-determining regions of the two target enzymes DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) leading to resistance occurs in a stepwise fashion. Whereas complete fluoroquinolone (FQ) resistance mainly requires mutations in both target enzymes, single mutations in only one of the target enzymes (first-step mutants) frequently are associated with intermediate resistance or susceptibility (9). Once a mutation in one of the target enzymes is present, there is a significantly increased likelihood for the acquisition of mutations in the second target enzyme, leading to complete resistance (7). Therefore, FQ treatment of infections caused by first-step mutants can lead to the selection of resistant isolates, resulting in treatment failure and a general increase in FQ resistance (3). First-step mutants cannot however be reliably detected by routine resistance testing.

In this study we applied a recently described PCR-restriction fragment length polymorphism (RFLP) assay to screen 496 susceptible invasive pneumococcal isolates collected by the Centers for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance (ABCS) Team (1). Only mutations which have been clearly demonstrated to confer resistance were considered (mutations S81X and E85K in *gyrA*; S79X and D83X in *parC*; and D435X in *parE*) (7, 13, 14).

Sterile-site isolates were collected from 1998 to 2003 by the CDC as part of the ABCS study. Randomly selected isolates were chosen using the randomization function of SPSS 11.0 (SPSS, Inc., Chicago, Ill.). Methods for case identification and isolate collection have been previously described (16). Serotyping and pulsed-field gel electrophoresis (PFGE) was performed as described previously (8, 10, 12). MICs were determined by broth microdilution carried out according to CLSI (formerly NCCLS) guidelines (11). The presence of an efflux pump was investigated by determination of the MICs of ciprofloxacin by the agar dilution method in the presence of reserpine (10 mg/liter) (2). The PCR-RFLP assay was performed as described by Alonso et al. (1). All isolates with suspected mutations were sequenced for confirmation.

A random sample of 286 of 17,328 isolates with a levofloxacin (LFX) MIC of 1 mg/liter, collected between 1998 and 2003 (Table 1), were analyzed. Only 1 of the 286 isolates harbored a mutation known to confer FQ resistance, resulting in an overall prevalence of 0.35%. The first-step mutant (serotype 19A) with an S79F alteration in *parC* was recovered from the blood of a 78-year-old long-term-care facility (LTCF) resident with pneumonia and diabetes. Since the prevalence of first-step mutants among invasive isolates with an LFX MIC of 1 mg/liter was low, we screened 84 isolates randomly selected from all isolates with an LFX MIC of 2 mg/liter collected from 1998 to 2001 (n = 1,139) and all available isolates with an LFX MIC of 2 mg/liter collected in 2002 (30 of 87) and 2003 (28 of 113). Eleven of these 142 isolates (7.7%) harbored a first-step mutation. Analysis of the demographic data of these 11 isolates revealed that LTCF residency could be a possible risk factor for the infection with first-step mutants; 2 of 10 (20%) first-step mutants (for one isolate with a first-step mutation, information about LTCF residence was not available) were from LTCF residents, while LTCF residents represented only 8.5% of the study population. In order to estimate the prevalence of first-step mutations in isolates from LTCF residents, we investigated all available ABCS isolates with an LFX MIC of 2 mg/liter collected from LTCF residents from 1998 to 2003 (n = 74). Twelve of these 74 isolates (16.2%) had a first-step mutation. This prevalence was significantly higher (chi-square test, $P = 0.026$) than that for the population outside the LTCF (8 of 125, 6.4%). Age has been described as a risk factor for fluoroquinolone resistance, and age may also be a risk factor for infection with first-step mutants. The LTCF residents in our study population had a mean age (± standard deviation) of 74 ± 17 years, compared to 50 ± 25 years for the non-LTCF residents (Mann-Whitney test, $P < 0.0001$). Including only

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TABLE 1. Levofloxacin MIC distribution of the ABCS collection by year

Yr	No. (%)[a] of isolates with LFX-MIC (mg/liter) of:					
	0.5	1	2	4	8	16
1998	272 (7.8)	2,743 (79.0)	450 (13.0)	1 (0.0)	1 (0.0)	5 (0.1)
1999	685 (17.0)	3,067 (76.0)	276 (6.8)	1 (0.0)	0 (0.0)	9 (0.2)
2000	363 (9.1)	3,288 (82.4)	326 (8.2)	0 (0.0)	4 (0.1)	9 (0.2)
2001	397 (11.2)	3,047 (85.6)	87 (2.5)	0 (0.0)	4 (0.1)	23 (0.7)
2002	521 (16.1)	2,610 (80.7)	87 (2.7)	0 (0.0)	3 (0.1)	12 (0.4)
2003	531 (16.4)	2,573 (79.7)	113 (3.5)	2 (0.1)	2 (0.1)	9 (0.3)

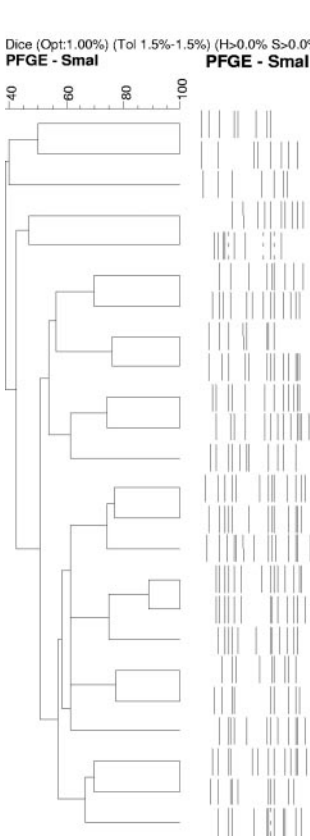
[a] Numbers in parentheses are cumulative percentages.

patients older than 35 years and controlling for age, we used logistic regression to test whether residency in an LTCF was an independent risk factor for infection with first-step mutants. It was found that LTCF residency was not significantly associated with infection with first-step mutants when controlling for age and comorbidities.

Fifteen of the 20 isolates with a first-step mutation exhibited a mutation in *parC* (Fig. 1). Of note, we found two isolates with a single *gyrA* alteration. PFGE of all identified first-step mutants revealed that most of the isolates were not closely related. Five of the 26 international Pneumococcal Molecular Epidemiology Network (PMEN) clones, which have already been shown to account for FQ resistance in the United States, were included in the PFGE analysis (15). Only 5 of 20 isolates (25%) could be assigned to one of the international clones. One cluster was related to the Spain^{23F}-1 clone, both isolates being recovered from LTCF residents in Connecticut. In addition, three single isolates were assigned to the England^{14}-9, Tennessee^{19}-18, and Taiwan^{23F}-15 clones, respectively. There was evidence for efflux in one-third of the isolates. We did not find a trend in efflux with regard to year, LFX MIC, age, or LTCF residency.

Data about the prevalence of first-step mutants are rare but these mutants are thought to be more common than resistant strains (6, 9). Our study revealed that the prevalence among invasive pneumococcal isolates with an LFX MIC of 1 mg/liter was insignificant. In contrast, it was 8% in invasive isolates with an LFX MIC of 2 mg/liter and as high as 16% among those strains from patients in LTCFs. There is an ongoing debate about the necessity for molecular susceptibility testing for FQ first-step mutants (4, 5, 9). Our data suggest that this might be of benefit for isolates with an LFX MIC of 2 mg/liter. Since sequencing might not be established easily as a routine technique, the PCR-RFLP assay used in this study provides valid results in a short time.

PFGE analysis revealed that to date there is little evidence for clonal spread of first-step mutants. An investigation of invasive LFX-resistant pneumococcal isolates which were collected from the same surveillance study revealed that about



ID	Serotype	State	Patient age	LFX MIC*	CIP MIC*	CIP+Res MIC*	*gyrA*	*parC*	*parE*
1043	16F	NY	81	2	4	2	WT	S79F	WT
835	6A	CT	58	2	4	2	WT	WT	D435H, I460V
843	8	OR	44	2	2	2	WT	D83G	WT
825	4	CO	46	2	4	2	WT	WT	D435H, I460V
831	14	CT	78	2	2	2	WT	S79Y	WT
1039	23F	TN	86	2	4	4	WT	S79F	WT
824	9V	MD	69	2	8	2	WT	S79Y, K137N	WT
1042	14	GA	80	2	4	2	WT	D83H	WT
Tenn14-18	14	Tennessee		1			WT	WT	WT
1045	33F	NY	72	2	2	1	S81Y	WT	WT
844	11A	MD	42	2	2	2	WT	D83G	WT
1034	6A	CT	79	2	4	2	WT	WT	D435N
817	23F	CT	70	2	4	2	WT	S79F	WT
Spain23F-1	23F	Spain		2			WT	K137N	I460V
1035	23F	CT	61	2	4	2	WT	S79F	WT
1033	14	CT	90	2	4	2	WT	S79F	WT
England14-9	14	UK		0.75			WT	WT	WT
789	19A	GA	78	1	4	2	WT	S79F	WT
837	23F	MD	83	2	8	2	WT	S79Y	WT
Taiwan23F-15	23F	Taiwan		0.75			WT	WT	WT
838	3	MD	38	2	4	2	S81F	WT	WT
1029	8	NY	88	2	2	1	WT	S79F	WT
1040	20	OR	90	2	8	2	WT	S79F, K137N	WT
815	19F	MD	44	2	4	2	WT	S79Y	WT

FIG. 1. Genetic relatedness of and mutations carried by first-step mutants among LFX-susceptible invasive isolates in the United States. Highlighted boxes display isolates related to one of the PMEN clones. Patient age is expressed in years. Abbreviations: ID, identifier; UK, United Kingdom; CIP, ciprofloxacin; CIP+Res, ciprofloxacin plus reserpine; WT, wild type. *, MIC in mg/liter.

half of the isolates could be assigned to one of the international PMEN clones (15). In contrast, in this study only 25% of the first-step mutants were related to one of the international clones.

First-step mutants are precursors of fully FQ-resistant strains. They are more frequent than resistant strains, and their propensity to acquire a second mutation argues that exposure of these strains to fluoroquinolones will continue to select for fluoroquinolone resistance. Continued surveillance of first-step mutants is necessary, particularly in LTCFs.

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REFERENCES

1. **Alonso, R., M. Galimand, and P. Courvalin.** 2004. An extended PCR-RFLP assay for detection of parC, parE and gyrA mutations in fluoroquinolone-resistant Streptococcus pneumoniae. J. Antimicrob. Chemother. **53:**682–683.
2. **Brenwald, N. P., M. J. Gill, and R. Wise.** 1998. Prevalence of a putative efflux mechanism among fluoroquinolone-resistant clinical isolates of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **42:**2032–2035.
3. **Croisier, D., M. Etienne, E. Bergoin, P. E. Charles, C. Lequeu, L. Piroth, H. Portier, and P. Chavanet.** 2004. Mutant selection window in levofloxacin and moxifloxacin treatments of experimental pneumococcal pneumonia in a rabbit model of human therapy. Antimicrob. Agents Chemother. **48:**1699–1707.
4. **Davies, T. A., A. Evangelista, S. Pfleger, K. Bush, D. F. Sahm, and R. Goldschmidt.** 2002. Prevalence of single mutations in topoisomerase type II genes among levofloxacin-susceptible clinical strains of *Streptococcus pneumoniae* isolated in the United States in 1992 to 1996 and 1999 to 2000. Antimicrob. Agents Chemother. **46:**119–124.
5. **Davies, T. A., and R. Goldschmidt.** 2002. Screening of large numbers of Streptococcus pneumoniae isolates for mutations associated with fluoroquinolone resistance using an oligonucleotide probe assay. FEMS Microbiol. Lett. **217:**219–224.
6. **Doern, G. V., S. S. Richter, A. Miller, N. Miller, C. Rice, K. Heilmann, and S. Beekmann.** 2005. Antimicrobial resistance among Streptococcus pneumoniae in the United States: have we begun to turn the corner on resistance to certain antimicrobial classes? Clin. Infect. Dis. **41:**139–148.
7. **Gillespie, S. H., L. L. Voelker, J. E. Ambler, C. Traini, and A. Dickens.** 2003. Fluoroquinolone resistance in Streptococcus pneumoniae: evidence that gyrA mutations arise at a lower rate and that mutation in gyrA or parC predisposes to further mutation. Microb. Drug Resist. **9:**17–24.
8. **Lefevre, J. C., G. Faucon, A. M. Sicard, and A. M. Gasc.** 1993. DNA fingerprinting of *Streptococcus pneumoniae* strains by pulsed-field gel electrophoresis. J. Clin. Microbiol. **31:**2724–2728.
9. **Lim, S., D. Bast, A. McGeer, J. de Azavedo, and D. E. Low.** 2003. Antimicrobial susceptibility breakpoints and first-step parC mutations in Streptococcus pneumoniae: redefining fluoroquinolone resistance. Emerg. Infect. Dis. **9:**833–837.
10. **McDougal, L., J. Rasheed, J. Biddle, and F. Tenover.** 1995. Identification of multiple clones of extended-spectrum cephalosporin-resistant *Streptococcus pneumoniae* isolates in the United States. Antimicrob. Agents Chemother. **39:**2282–2288.
11. **NCCLS.** 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically—approved standard M7-A6, 6th ed. NCCLS, Wayne, Pa.
12. **Neufeld, F.** 1902. Über die Agglutination der Pneumokokken und über die Theorie der Agglutination. Z. Hyg. Infektionskr. **34:**54–72.
13. **Pan, X. S., J. Ambler, S. Mehtar, and L. M. Fisher.** 1996. Involvement of topoisomerase IV and DNA gyrase as ciprofloxacin targets in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **40:**2321–2326.
14. **Perichon, B., J. Tankovic, and P. Courvalin.** 1997. Characterization of a mutation in the *parE* gene that confers fluoroquinolone resistance in *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. **41:**1166–1167.
15. **Pletz, M. W., L. McGee, J. Jorgensen, B. Beall, R. R. Facklam, C. G. Whitney, and K. P. Klugman.** 2004. Levofloxacin-resistant invasive *Streptococcus pneumoniae* in the United States: evidence for clonal spread and the impact of conjugate pneumococcal vaccine. Antimicrob. Agents Chemother. **48:**3491–3497.
16. **Whitney, C. G., M. M. Farley, J. Hadler, L. H. Harrison, C. Lexau, A. Reingold, L. Lefkowitz, P. R. Cieslak, M. Cetron, E. R. Zell, J. H. Jorgensen, and A. Schuchat.** 2000. Increasing prevalence of multidrug-resistant Streptococcus pneumoniae in the United States. N. Engl. J. Med. **343:**1917–1924.