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Molecular Characterization and Antimicrobial Susceptibility of Fluoroquinolone-Resistant or -Susceptible *Streptococcus pneumoniae* from Hong Kong

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Fluoroquinolone resistance in *Streptococcus pneumoniae* isolated from Hong Kong as part of Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin 1999/2000 was found to be due to the spread of the Spain^{23F}-1 clone (mainly a Spain^{23F}-1-14 variant). All the isolates were multidrug resistant but were susceptible to quinupristin-dalfopristin, linezolid, and telithromycin. The Spain^{23F}-1 clone also occurred among antimicrobial-susceptible isolates, which suggests that the primary source of this clone may have been Asia rather than Iberia.

The global prevalence of fluoroquinolone resistance among *Streptococcus pneumoniae* is low at around 1% (4, 5, 9, 13). However, "hot spots" with considerably higher fluoroquinolone resistance rates do occur. Ho and colleagues have previously described high levels of fluoroquinolone resistance in pneumococci from Hong Kong due to well-established mutations in topoisomerase IV and DNA gyrase (6, 7). The same research group, using isolates from a different period, found fluoroquinolone resistance in Hong Kong to be due to a single strain, the Spain^{23F}-1 clone, but the mechanism of fluoroquinolone resistance was not determined (8). Likewise, in the study Prospective Resistant Organism Tracking and Epidemiology for the Ketolide Telithromycin 1999/2000 (PROTEKT 1999/2000), high-level fluoroquinolone resistance was detected in Hong Kong—a total of 10 out of 70 *S. pneumoniae* isolates were levofloxacin resistant (14% resistance) (4).

We therefore analyzed the genetic relatedness and mechanism of fluoroquinolone resistance for all 10 fluoroquinolone-resistant pneumococci isolated from Hong Kong during the PROTEKT 1999/2000 study plus 38 additional fluoroquinolone-susceptible isolates collected during the same period.

(Preliminary data were presented at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy in 2002 [abstract C2-703]).

MIC was determined for a number of antibacterial agents based on the NCCLS broth microdilution method (11) by using dried microtiter plates supplied by TREK Diagnostics Ltd., East Grinstead, United Kingdom.

Type II DNA topoisomerase genes *gyrA*, *gyrB*, *parC*, and *parE* were amplified by using DNA extracted with a High Pure PCR Template Kit (Roche, Lewes, United Kingdom) as a template in 50-μl volumes with 2 units of Platinum *Taq* in reaction buffer supplied by the manufacturer (Invitrogen, Paisley, United Kingdom), with forward and reverse primers (described in Table 1) at a final concentration of 20 nmol/ml and $MgCl_2$ at a final concentration of 1.5 mM. PCR was carried out at an initial incubation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 3 min. This was followed by a final elongation step of 72°C for 10 min. Amplified DNA was stored at 4°C until required. Each DNA topoisomerase gene was fully sequenced by using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Warrington, United Kingdom) with additional internal primers.

Pulsed-field gel electrophoresis with *Sma*I digestion and multilocus sequence typing (MLST) was also carried out as described previously (1, 2). MLST analysis was compared with isolates logged onto the MLST database (http://www.mlst.net). Only fluoroquinolone-resistant pneumococci were serotyped by using standard methods and sera with various reactivities from the Statens Serum Institute (Copenhagen, Denmark).

The antibacterial agent susceptibility of the 10 fluoroquinolone-resistant isolates is shown in Table 2. According to NCCLS breakpoints (12), nine were penicillin G resistant and one was penicillin G intermediate. All the isolates were resistant to erythromycin A, tetracycline, and sulfamethoxazole-trimethoprim (i.e., multidrug resistant) but remained susceptible to telithromycin (no NCCLS breakpoint is presently available, but the highest MIC obtained was 0.06 μg/ml), quinupristin-dalfopristin, and linezolid.

It was found that all 10 fluoroquinolone-resistant isolates belonged to the same multilocus sequence type 81, this being

TABLE 1. Primers used to amplify regions of pneumococcal genomic DNA contaning type II topoisomerase genes

Description	Designation	Sequence (5′ to 3′)
gyrA forward primer	SPGYRAF	GAGGCTGAAATAGATGGGAATT
gyrA reverse primer	SPGYRAR	CTGCTAGGATATTTGTCAGC
gyrB forward primer	SPGYRBF	CCAGTATCGTGAATTTAAGACA
gyrB reverse primer	SPGYRBR	TCCATTAGACATATCAAACTCC
parC forward primer	SPPARCF	TTGTGAGTTTGCTTACTTCAAG
parC reverse primer	SPPARCR	GTCATATTCCACTCCTTATTC
parE forward primer	SPPAREF	AGAGAATCTCTGCTGAAATTGT
parE reverse primer	SPPARER	CACATAGTCATTCACATCCGA

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TABLE 2. Antimicrobial susceptibility of 10 fluoroquinolone-resistant *S. pneumoniae* isolates from Hong Kong

Isolate no.	MIC (μg/ml)									
	Ciprofloxacin	Levofloxacin	Moxifloxacin	Penicillin G	Erythromycin	Tetracycline	Sulfamethoxazole-Trimethoprim	Telithromycin	Quinupristin-Dalfopristin	Linezolid
P1060004	>32	16	>4	4	>64	>16	>16	0.03	0.5	1
P1060012	>32	16	4	4	>64	>16	8	0.03	0.5	1
P1060023	>32	16	4	4	>64	>16	>16	0.03	0.5	1
P1060024	>32	16	4	4	>64	>16	>16	0.03	0.5	1
P1060027	>32	16	4	4	>64	16	16	0.06	0.5	1
P1060040	>32	16	4	4	>64	>16	>16	0.06	0.5	1
P1060003	>32	32	4	4	2	>16	8	0.06	0.25	1
P1060045	>32	16	4	2	>64	>16	>16	0.06	0.5	1
P1060053	>32	16	4	2	>64	16	8	0.03	0.5	1
P1060195	>32	16	4	1	>64	>16	8	0.03	0.25	1

the Spain^{23F}-1 clone. Interestingly, nine of these isolates expressed the serotype 14 capsule and should therefore be described more accurately as Spain^{23F}-1-14 (10). These isolates possessed an identical Ser-79-Phe alteration in ParC in combination with Ser-81-Phe in GyrA. Only strain P1060003 retained the "original" serotype 23F capsule and showed a closely related pulsed-field gel electrophoresis pattern that was nonetheless different from that seen with the other fluoroquinolone-resistant isolates (results not shown). Furthermore, P1060003 possessed an additional Asp-435-Asn alteration in ParE and the Ser-81 substitution in GyrA was to Tyr rather than Phe. The possession of three topoisomerase alterations may explain the higher levofloxacin MIC obtained with this isolate (Table 2). P1060003 also differed from the other Spain^{23F}-1clones because it possessed low-level erythromycin A resistance (Table 2), which was determined in a separate study to be due to the macrolide efflux gene *mef*(A), whereas the other nine fluoroquinolone-resistant isolates possess *erm*(B) (3). These data suggest, therefore, that there were only two clonal types associated with fluoroquinolone resistance in Hong Kong, with the Spain^{23F}-1-14 variant being most dominant. This confirms the results of Ho et al. (8). We have no way of knowing for sure that identical mutations did not occur independently in each separate isolate of the Spain^{23F}-1 lineage, but, as the mutation rate to fluoroquinolone resistance is perceived as being very low, it would seem most likely that the high resistance in Hong Kong was due to clonal spread. This means that the true mutation rate in Hong Kong may in fact have been lower than 1 in 7 and was possibly nearer to 2 in 70 (2.9%). Furthermore, what has happened in Hong Kong may be unique to that particular community because the development of fluoroquinolone resistance in the global Spain^{23F}-1 clone or variants has not been described elsewhere to date.

When MLST was determined for the 38 fluoroquinolone-susceptible *S. pneumoniae* isolates (Table 3), 14 of these isolates had MLST profiles that matched the Spain^{23F}-1 clone (MLST 81) and one was a single-locus variant of this clone (with three nucleotide changes in the *gdh* gene). The remaining isolates were from a wide range of separate clonal lineages, some of which have not been described before (Table 3). Surprisingly, two of the Spain^{23F}-1 clones (P1060002 and P1060200) were not multidrug resistant (Table 4). One other Spain^{23F}-1 clone (P1060028) was also susceptible to penicillin G but was highly resistant to erythromycin A. As far as we are aware, this study is the first to describe penicillin G- and erythromycin A-susceptible isolates of the Spain^{23F}-1 clone. These data also suggest that, despite the nomenclature, the Spain^{23F}-1 clone may not have originated in Spain but in Hong

TABLE 3. Multilocus sequence typing characteristics of 38 fluoroquinolone-susceptible *S. pneumoniae* isolates from Hong Kong

Matching clone[a]	No. detected	MLST allele no.							Multilocus sequence type
		aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	
MDR Spain^{23F}-1	14	4	4	2	4	4	1	1	81
Serotype 3 (susceptible)	5	7	15	2	10	6	1	22	180
MDR France/Spain$^{9V/14}$-1	3	7	11	10	1	6	8	1	156
England^{14}-9	2	1	5	4	5	5	1	8	9
UK$^{19A/15B}$-1 (susceptible)	2	8	13	14	4	17	4	14	199
SLV of Vietnam isolate	2	10	17	4	16	6	1	17	New
Oxford^{23F}-1 (susceptible)	1	1	8	9	1	6	4	6	311
MDR Taiwan^{23F}-1	1	15	29	4	21	30	1	14	242
MDR Taiwan^{19F}-1	1	15	16	19	15	6	20	26	236
SLV of Spain^{23F}-1	1	4	15	2	4	4	1	1	New
2LV of Taiwan^{8}-1	1	15	N	34	16	9	14	N	New
No close matches	1	5	31	4	1	9	N	5	New
No close matches	1	15	22	2	1	6	14	17	New
No close matches	1	7	N	9	6	25	6	14	New
No close matches	1	5	5	N	1	6	1	8	New
No close matches	1	8	10	2	16	2	N	59	New

[a] Abbreviations: MDR, multidrug resistant; SLV, single-locus variant; 2LV, two-locus variant; N, not described in MLST database.

TABLE 4. Antimicrobial susceptibility of the 14 MLST 81 and 1 MLST 81 single-locus variant fluoroquinolone-susceptible *S. pneumoniae* isolates from Hong Kong

Isolate no.	MIC (μg/ml)									
	Penicillin G	Erythro-mycin	Tetra-cycline	Sulfamethoxazole-Trimethoprim	Telithro-mycin	Quinupristin-Dalfopristin	Linezolid	Cipro-floxacin	Levo-floxacin	Moxi-floxacin
P1060030	4	4	16	4	0.12	0.5	1	1	1	0.12
P1060014	4	2	>16	16	0.06	0.5	2	2	1	0.25
P1060020	4	2	>16	>16	0.06	0.5	1	2	1	0.25
P1060031	4	2	>16	>16	0.06	0.5	1	2	1	0.25
P1060036	4	2	>16	8	0.06	0.5	1	2	1	0.12
P1060009	4	2	>16	8	0.06	0.5	2	1	1	0.25
P1060035	4	2	>16	8	0.06	0.5	1	1	1	0.12
P1060198	2	4	>16	16	0.06	0.25	1	1	1	0.12
P1060197	2	2	>16	8	0.06	0.5	1	2	1	0.12
P1060018	2	2	>16	>16	0.6	0.5	1	1	1	0.25
P1060057	2	1	>16	16	0.03	0.25	1	2	1	0.12
P1060050	2	1	>16	8	0.03	0.5	1	1	1	0.12
P1060002	0.015	0.06	8	0.5	0.03	1	1	2	1	0.25
P1060200	0.015	0.06	8	0.5	0.008	0.5	1	1	1	0.12
P1060028[a]	≤0.008	>64	>16	2	0.5	0.5	1	2	2	0.5

[a] Single-locus variant of Spain^{23F}-1.

Kong. Furthermore, antibacterial-susceptible Spain^{23F}-1 may have become dominant in this region during the preantibiotic era and the ability of the Spain^{23F}-1 clone to spread may not be due to antibiotic resistance alone.

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