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Clonal Spread of Levofloxacin-Resistant *Streptococcus pneumoniae* Invasive Isolates in Madrid, Spain, 2007 to 2009[▽]

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Among 1,349 *Streptococcus pneumoniae* invasive isolates, 45 (3.3%) were levofloxacin resistant. Serotype distribution was as follows: 8 ($n = 32$ isolates), 19A ($n = 4$ isolates), 7F ($n = 3$ isolates), 9V ($n = 2$ isolates), 10A ($n = 1$ isolate), 19F ($n = 1$ isolate), 6B ($n = 1$ isolate), and nontypeable ($n = 1$ isolate). Levofloxacin-resistant isolates had dual mutations in the *gyrA* and *parC* genes. Serotype 8 strains corresponded to a capsular switching of the Sweden^{15A}-25 clone. Levofloxacin resistance was also detected among multiresistant (ST276^{19A}, Spain^{9V}-ST156, ST88^{19F}, and ST1542^{6B}) and among usually antibiotic-susceptible (Netherlands^{7F}-ST191, ST1201^{19A}, and ST2639^{10A}) clones.

Dissemination of non-antimicrobial-susceptible clones has been a major factor in the emergence of resistance in *Streptococcus pneumoniae* (10, 15). Moreover, the introduction of the pneumococcal 7-valent conjugate vaccine (PCV7) has brought about a shift (9, 13, 16), with an increase in the incidence of non-PCV7 nonsusceptible serotypes, such as 19A, which can complicate the selection of empirical treatments. Recently, the 13-valent pneumococcal conjugate vaccine (PCV13) has been granted marketing authorization. Currently, most of the circulating serotypes in Spain are covered by this vaccine (9).

Resistance to fluoroquinolones among pneumococci is primarily caused by mutations in the quinolone resistance-determining regions (QRDRs) of the *parC* and *gyrA* genes (7, 18, 20). Although at present the prevalence of fluoroquinolone resistance in *S. pneumoniae* remains low (5, 17), ongoing surveillance is necessary.

In this study, we describe the serotypes, antimicrobial susceptibilities, fluoroquinolone resistance mutations, and molecular characterizations of levofloxacin-resistant invasive pneumococci isolated in our area over a 2-year period.

From February 2007 to January 2009, a total of 1,349 invasive strains (one isolate for every invasive pneumococcal disease [IPD] episode) from 1,324 patients (age range, <1 to 101 years; 785 males) were studied. Nineteen patients had two recurrent infections, and three had three recurrences. The strains were recovered from 34 hospitals in the area of Madrid (Spain).

Identification of the capsular serotypes was determined by the Pneumotest-Latex kit (Statens Serum Institut, Copenhagen, Denmark) and by Quellung reaction using commercial factor antisera (Statens Serum Institut, Copenhagen, Denmark).

Susceptibilities to penicillin, erythromycin, clindamycin, cefotaxime, vancomycin, and levofloxacin were determined by the Etest method (AB bioMérieux, Solna, Sweden). Inducible resistance to macrolides was detected by the double-disk diffusion method using erythromycin (15 μg) and clindamycin (2 μg) disks.

Levofloxacin-resistant (MIC ≥ 8 μg/ml) *S. pneumoniae* isolates were characterized by pulsed-field gel electrophoresis (PFGE) after restriction with SmaI (14). Multilocus sequence typing (MLST) was performed as previously described (8). The QRDR regions of the *gyrA* and *parC* genes were amplified and sequenced as described elsewhere (18).

Among 1,349 *S. pneumoniae* invasive isolates, 45 levofloxacin-resistant strains (3.3%; all showing a levofloxacin MIC of ≥32 μg/ml) were detected. Only two strains (0.15%) showed intermediate susceptibility (levofloxacin MIC of 4 μg/ml). Overall, the MIC_{50} and MIC_{90} of levofloxacin were 0.75 and 1.75 μg/ml, respectively. All levofloxacin-resistant strains were isolated from adult patients (age range, 35 to 98 years). Serotype distribution was as follows: 8 ($n = 32$ isolates), 19A ($n = 4$ isolates), 7F ($n = 3$ isolates), 9V ($n = 2$ isolates), 10A ($n = 1$ isolate), 19F ($n = 1$ isolate), 6B ($n = 1$ isolate), and nontypeable ($n = 1$ isolate). Most serotype 8 levofloxacin-resistant strains ($n = 19$) were isolated from a single hospital (the remaining 13 strains were isolated from eight different hospitals).

Thirty-nine of the 45 levofloxacin-resistant strains, serotypes 8 ($n = 28$), 19A ($n = 4$), 7F ($n = 2$), 10A ($n = 1$), 19F ($n = 1$), 6B ($n = 1$), 9V ($n = 1$), and nontypeable ($n = 1$), were available for molecular characterization. Results of serotyping,

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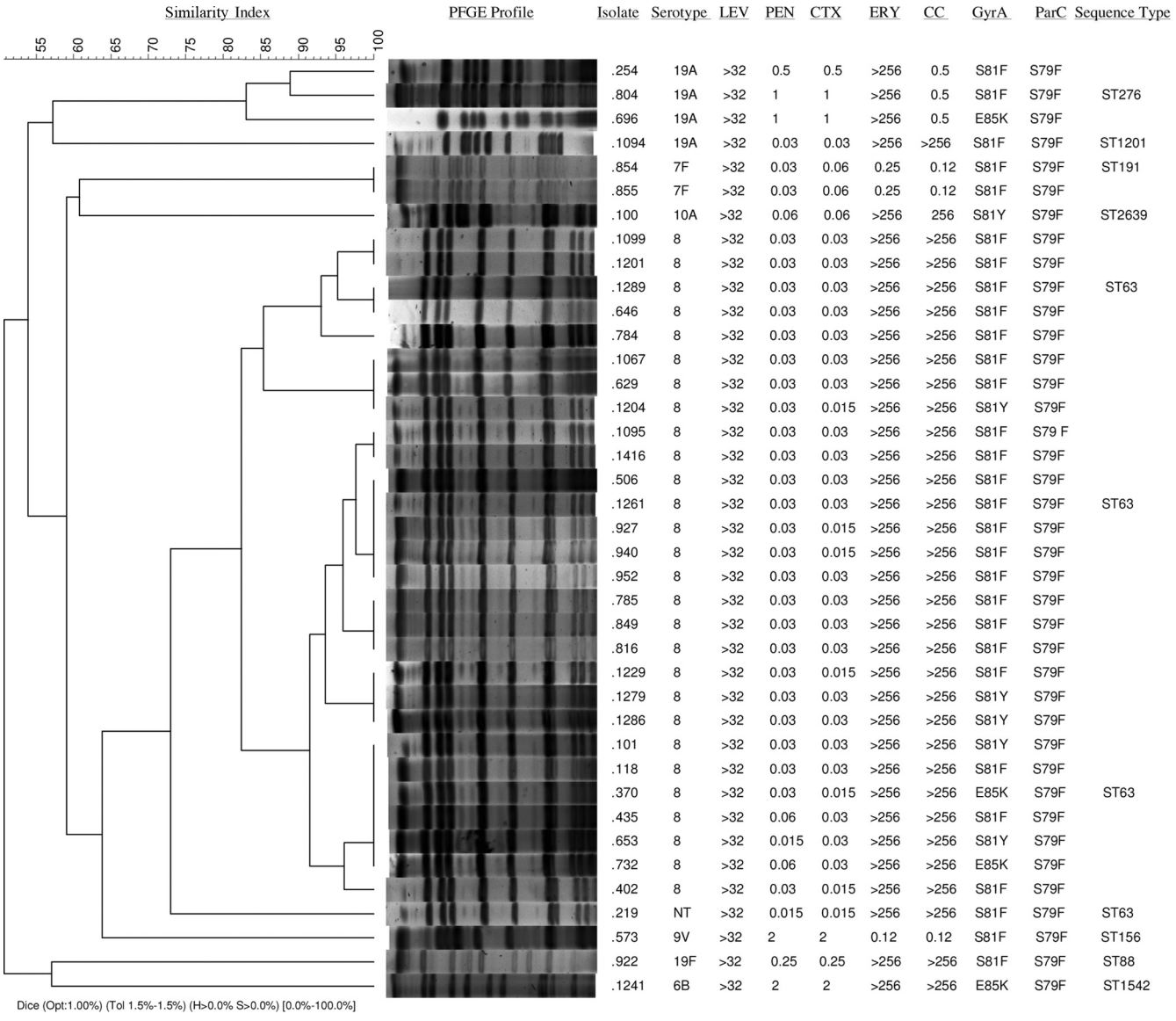

Isolate	Serotype	LEV	PEN	CTX	ERY	CC	GyrA	ParC	Sequence Type
.254	19A	>32	0.5	0.5	>256	0.5	S81F	S79F	
.804	19A	>32	1	1	>256	0.5	S81F	S79F	ST276
.696	19A	>32	1	1	>256	0.5	E85K	S79F	
.1094	19A	>32	0.03	0.03	>256	>256	S81F	S79F	ST1201
.854	7F	>32	0.03	0.06	0.25	0.12	S81F	S79F	ST191
.855	7F	>32	0.03	0.06	0.25	0.12	S81F	S79F	
.100	10A	>32	0.06	0.06	>256	256	S81Y	S79F	ST2639
.1099	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1201	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1289	8	>32	0.03	0.03	>256	>256	S81F	S79F	ST63
.646	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.784	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1067	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.629	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1204	8	>32	0.03	0.015	>256	>256	S81Y	S79F	
.1095	8	>32	0.03	0.03	>256	>256	S81F	S79 F	
.1416	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.506	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1261	8	>32	0.03	0.03	>256	>256	S81F	S79F	ST63
.927	8	>32	0.03	0.015	>256	>256	S81F	S79F	
.940	8	>32	0.03	0.015	>256	>256	S81F	S79F	
.952	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.785	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.849	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.816	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.1229	8	>32	0.03	0.015	>256	>256	S81F	S79F	
.1279	8	>32	0.03	0.03	>256	>256	S81Y	S79F	
.1286	8	>32	0.03	0.03	>256	>256	S81Y	S79F	
.101	8	>32	0.03	0.03	>256	>256	S81Y	S79F	
.118	8	>32	0.03	0.03	>256	>256	S81F	S79F	
.370	8	>32	0.03	0.015	>256	>256	E85K	S79F	ST63
.435	8	>32	0.06	0.03	>256	>256	S81F	S79F	
.653	8	>32	0.015	0.03	>256	>256	S81Y	S79F	
.732	8	>32	0.06	0.03	>256	>256	E85K	S79F	
.402	8	>32	0.03	0.015	>256	>256	S81F	S79F	
.219	NT	>32	0.015	0.015	>256	>256	S81F	S79F	ST63
.573	9V	>32	2	2	0.12	0.12	S81F	S79F	ST156
.922	19F	>32	0.25	0.25	>256	>256	S81F	S79F	ST88
.1241	6B	>32	2	2	>256	>256	E85K	S79F	ST1542

FIG. 1. PFGE dendrogram and information of 39 levofloxacin-resistant *S. pneumoniae* isolates, including serotype, MICs (μg/ml) of the antimicrobial agents tested, sequence type (ST), and QRDR substitutions in GyrA and ParC. LEV, levofloxacin; PEN, penicillin; CTX, cefotaxime; ERY, erythromycin; CC, clindamycin.

antimicrobial susceptibility, QRDR characterization, PFGE, and sequence type (ST) are shown in Fig. 1.

The 28 serotype 8 strains showed one identical PFGE type, were susceptible to penicillin and cefotaxime, and showed erythromycin and clindamycin resistance with the constitutive macrolide-lincosamide-streptogramin B (cMLSB) phenotype. Characterization of *gyrA* and *parC* QRDRs identified the ParC S79F mutation in all isolates. In addition, 21 isolates presented the S81F mutation, five the S81Y mutation, and two the E85K mutation in GyrA. Three randomly selected isolates from this PFGE type were shown to be ST63, which corresponds to a capsular switching event of the Sweden^{15A}-25-ST63 clone. The nontypeable strain was grouped in the same PFGE type as serotype 8 isolates, and MLST analysis identified this strain as belonging to ST63.

Among the four serotype 19A isolates, two PFGE types were identified, and one included three erythromycin-resistant and clindamycin-intermediate isolates, expressing the inducible MLSB (iMLSB) phenotype. All three isolates presented the ParC S79F mutation, and two of them harbored the S81F mutation and one the E85K mutation in GyrA. MLST of one of the three isolates identified the strain as ST276, a single-locus variant (SLV) of ST230, representative of the Denmark^{14}-32 clone. The remaining 19A isolate, identified as ST1201, was susceptible to penicillin, cefotaxime, erythromycin, and clindamycin and showed S79F ParC and S81F GyrA substitutions. The two serotype 7F isolates were shown to be ST191. The only 9V strain available was identified as ST156 (Spain^{9V}-3 clone). The 6B isolate was identified as ST1542, an SLV of Spain^{6B}-2-ST90. Isolates expressing 19F and 10A se-

rotypes were identified as ST88 (minor Spanish 19F clone) and ST2639, respectively.

In this study, the prevalence of fluoroquinolone resistance among pneumococcal invasive isolates was 3.3%, which is higher than previously reported in Spain (4). It has been proposed that most fluoroquinolone-resistant strains of *S. pneumoniae* arise from heterogeneous mutations (11, 12). However, levofloxacin resistance in this study was limited to a few serotypes and was due mainly to a clonal spread of a serotype 8 ST63 (Sweden^{15A}-25). These isolates were fully susceptible to penicillin and resistant to erythromycin and clindamycin. This clone, not included in the conjugate vaccine, has been previously detected among fluoroquinolone-resistant *S. pneumoniae* in Spain (4), and capsular switching events have also been described in this clone (4). The serotype 19A ST276 clone, an SLV of Denmark^{14}-32-ST230, was also responsible for resistance to levofloxacin in our series. This multiresistant clone included in the PCV13 is disseminated worldwide (9, 13, 16). However, to our knowledge, this is the first report of resistance to levofloxacin in strains belonging to this clone. In addition, it is noteworthy that we have detected resistance to fluoroquinolones among usually antibiotic-susceptible clones circulating in Spain: Netherlands^{7F}-ST191, ST1201 (19A), and ST2639 (10A) (1, 6, 19).

DNA sequence analysis of the QRDRs of the *gyrA* and *parC* genes showed that all isolates contained the S79F mutation in ParC. All isolates also presented amino acid changes in GyrA, with most changes occurring at S81, whereas changes at E85 were rare. We found heterogeneity in the GyrA QRDR amino acid substitutions among isolates belonging to the same PFGE type. This suggests that dissemination of these organisms is not only due to clonal spread but probably also due to independent selection.

Strains with first-step mutation to quinolones (mutation in only one of the target genes) are at a higher risk for developing resistance (2) and may determine the treatment outcome (3). However, the very low proportion of strains with intermediate susceptibility to levofloxacin detected in this study (0.15%) suggests that isolates with first-step mutations to quinolones are not very frequent in our area.

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