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# Fluoroquinolone-Resistant Pneumococci: Dynamics of Serotypes and Clones in Spain in 2012 Compared with Those from 2002 and 2006

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**In Spain, rates of ciprofloxacin resistance in pneumococci were low during the last decade (2.6% in 2002 and 2.3% in 2006). In 2012, the rate remained at 2.3%, equivalent to 83 of 3,621 isolates. Of the 83 resistant isolates, 15 showed a low level (MIC of 4 to 8 μg/ml) and 68 a high level (MIC of 16 to 128 μg/ml) of ciprofloxacin resistance. Thirteen low-level-resistant isolates had single changes in ParC, one had a single ParE change, and one did not present any mutations. High-level-resistant isolates had GyrA changes plus additional ParC and/or ParE changes: 51, 15, and 2 isolates had 2, 3, or 4 mutations, respectively. Although 24 different serotypes were observed, 6 serotypes accounted for 51.8% of ciprofloxacin-resistant isolates: 8 (14.5%), 19A (10.8%), 11A (7.2%), 23A (7.2%), 15A (6.0%), and 6B (6.0%). A decrease in pneumococcal 7-valent conjugate vaccine (PCV7) serotypes was observed from 2006 (35.7%) to 2012 (16.9%), especially of serotype 14 (from 16.3% to 2.4%; $P < 0.001$). In comparison with findings in 2006, multidrug resistance was greater in 2012 ($P = 0.296$), mainly due to the increased presence and/or emergence of clonal complexes associated with non-PCV7 serotypes: CC63 expressing serotypes 8, 15A, and 19A; CC320 (with serotype 19A); and CC42 (with serotype 23A). Although rates of ciprofloxacin resistance remained low and stable throughout the last decade, changes in serotype and genotype distributions were observed in 2012, notably the expansion of a preexisting multidrug-resistant clone, CC63, and the emergence of the CC156 clone expressing serotype 11A.**

*Streptococcus pneumoniae* is an important cause of morbidity and mortality worldwide, and it is a major etiological agent of community-acquired pneumonia, meningitis, and acute otitis media (1). Following the introduction of the pneumococcal 7-valent conjugate vaccine (PCV7) in 2000 in the United States, the incidence of invasive pneumococcal disease declined drastically, coinciding with a decrease in penicillin resistance (2–4). In Spain, where PCV7 was introduced in 2001, a decrease in the incidence of invasive disease due to PCV7 serotypes was also observed (5). However, shortly after introduction of PCV7, an emergence of nonvaccine serotypes was observed worldwide (6, 7).

Fluoroquinolones (FQs) target type II DNA topoisomerases. Despite the functional similarities between topoisomerase IV (topo IV) and gyrase, their susceptibilities to FQs vary across bacterial species (8). Isolates of *S. pneumoniae* that are resistant to FQs have been shown to present mutations at specific regions (quinolone resistance determining regions [QRDRs]) of the topoisomerase IV (*parC* and *parE*) and DNA gyrase (*gyrA*) genes. In recent decades, the new-generation FQs levofloxacin (LVX) and moxifloxacin (MOX), which have enhanced activity against pneumococci and other respiratory pathogens, have become therapeutic alternatives in the treatment of community-acquired pneumonia. In *S. pneumoniae*, the primary target for ciprofloxacin (CIP) and LVX is topo IV (9–12), whereas gyrase is the primary target for MOX (13). Although CIP has low activity against *S. pneumoniae* and is not recommended for treatment, it has proved to be useful for detection of first-step mutations. In the present study, FQ resistance was considered when the CIP MIC was ≥4 μg/ml, following the criteria established by Chen et al. (14), which coincides with the current (>2 μg/ml) EUCAST breakpoints (15). The differences observed in the rates of susceptibility to CIP compared with those to LVX and MOX are due to isolates with first-step QRDR mutations. These isolates (CIP resistant but LVX or MOX susceptible) could become highly resistant under selective FQ pressure and are associated with treatment failure when FQs are used (16). By using a CIP resistance breakpoint MIC of ≥4 μg/ml, we have detected first-step mutations in isolates susceptible to LVX by the CLSI criteria (LVX MIC of 1 to 2 μg/ml). In addition, among isolates with a CIP MIC of 2 μg/ml, no first-step mutations were detected in our previous studies (17, 18). The killing effect of FQs has been related to the resolution of reaction intermediates of DNA-FQ-topoisomerase complexes, which subsequently generates irreparable double-stranded DNA breaks (19).

CIP resistance in *S. pneumoniae* continues to show a low prevalence (<3%) in Europe (18, 20), although higher rates have been detected in Asia (10.5%) (21) and Canada (7.3%) (22). Resistance to FQs can evolve during treatment, and there are numerous reports of treatment failures with the use of FQs in pneumococcal infections caused by strains with first-step mutations (14, 23). These cases tend to involve elderly patients with chronic respiratory diseases, such as chronic obstructive pulmonary disease (COPD), in which higher rates of FQ resistance have been detected (24). Although the development of FQ resistance has been associated with FQ consumption (14, 22, 25), the dissemination of pneumococcal FQ-resistant clones has rarely been observed so far (26). However, previous epidemiological studies (17, 18) have re-

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**TABLE 1** Comparison of three surveillance studies of ciprofloxacin-resistant *Streptococcus pneumoniae* isolates in Spain: data for 2002, 2006, and 2012[a]

| Characteristic | No. of Cip$^r$ isolates/total no. of isolates (%)[c] | | | *P* value[d] | |
|---|---|---|---|---|---|
| | 2002 | 2006 | 2012 | 2002 vs 2012 | 2006 vs 2012 |
| Ciprofloxacin resistance | | | | | |
| Global | 75/2,882 (2.6) | 98/4,215 (2.3) | 83/3,621 (2.3) | 0.419 | 0.940 |
| Low level (MICs of 4–8 μg/ml) | 14/75 (18.7) | 30/98 (30.6) | 15/83 (18.1) | 1.000 | 0.059 |
| High level (MICs ≥ 16 μg/ml) | 61/75 (81.3) | 68/98 (69.3) | 68/83 (/81.9) | 1.000 | 0.059 |
| In persons <15 years of age | 0/978 (0) | 2/1,446 (0.14) | 2/695 (0.4) | 0.172 | 0.600 |
| In persons 15–64 years of age | 22/1,166 (1.9) | 34/1,455 (2.3) | 19/1,336 (1.4) | 0.431 | 0.100 |
| In persons >64 years of age | 53/738 (7.2) | 61/1,314 (4.7) | 62/1,590 (3.9) | **<0.001** | 0.355 |
| PCV7 serotypes | 49/75 (65.3) | 35/98 (37.5) | 14/83 (16.9) | **<0.001** | **0.014** |
| PCV13 serotypes | 56/75 (74.7) | 47/98 (48.0) | 31/83 (37.3) | **<0.001** | 0.176 |
| Serotype 8 | 0/75 (0) | 7/98 (7.1) | 12/83 (14.5) | **<0.001** | 0.145 |
| Serotype 11A | 1/75 (1.3) | 4/98 (4.1) | 6/83 (7.2) | 0.120 | 0.516 |
| Serotype 14 | 14/75 (18.7) | 16/98 (16.3) | 2/83 (2.4) | **<0.001** | **<0.001** |
| Serotype 19A | 1/75 (1.3) | 8/98 (8.2) | 9/83 (10.8) | **0.019** | 0.613 |
| Other antimicrobial drug resistance | | | | | |
| Penicillin MIC ≥ 0.12 μg/ml | 55/75 (73.3) | 44/98 (44.9) | 37/83 (44.5) | **<0.001** | 1.000 |
| Chloramphenicol MIC ≥ 8 μg/ml | 33/75 (44.0) | 11/98 (11.2) | 4/83 (4.8) | **<0.001** | 0.176 |
| Multidrug resistance[b] | 55/75 (73.3) | 48/98 (49.0) | 48/83 (57.8) | **0.046** | 0.296 |

[a] Cip$^r$, resistant to ciprofloxacin, defined by Chen et al. as ≥4 μg/ml (22); low-level resistant, MICs of 4 to 8 μg/ml; high-level resistant, MICs ≥ 16 μg/ml.
[b] Multidrug resistance, resistance to CIP and to at least two other antimicrobial groups.
[c] Values for "Other antimicrobial drug resistance" are no. of resistant isolates/no. of ciprofloxacin-resistant isolates (%).
[d] Values in bold indicate statistically significant differences ($P < 0.05$) between time periods.

vealed a low genetic diversity of pneumococcal clones among FQ-resistant pneumococci in Spain.

The present study investigates the prevalence of FQ-resistant pneumococci in Spain during 2012. Resistance mutations in the QRDRs of *parC*, *parE*, and *gyrA* were studied, as were resistance associations with other antibiotics and the characteristics of the clones harboring this resistance. In order to assess changes in the epidemiology of FQ resistance, the results of the present study were compared with those of two similar studies conducted in 2002 and 2006.

## MATERIALS AND METHODS

**Bacterial isolates, serotyping, and susceptibility tests.** A total of 3,621 *S. pneumoniae* isolates from 112 hospitals nationwide were sent to the Spanish Pneumococcus Reference Laboratory during 2012: 2,926 isolates were from adults, and 695 were from children. In terms of their origins, 2,252 (62.2%) isolates were from blood or other sterile sites, while the remaining 1,369 (37.8%) were from respiratory samples. Isolates were confirmed as *S. pneumoniae* by standard methods, with serotypes being determined by the Quellung reaction. Antimicrobial susceptibility was tested by agar dilution at the Spanish Reference Laboratory. The MICs of CIP, LEV, and MOX of 83 isolates with CIP MICs of ≥4 μg/ml were confirmed by Etest and broth microdilution methods, according to the Clinical and Laboratory Standards Institute guidelines (27). *S. pneumoniae* ATCC 49619 was included as a quality control.

**Molecular typing.** Clonal complexes (CCs) were characterized by means of pulsed-field gel electrophoresis (PFGE). Briefly, genomic DNA embedded in agarose plugs was restricted with SmaI or ApaI, and fragments were separated by PFGE in a Chef-DRIII apparatus (Bio-Rad). PFGE patterns were visually compared with those of representative international pneumococcal clones of the Pneumococcal Molecular Epidemiology Network (28), and isolates with patterns that varied by three or fewer bands were considered to represent the same PFGE type. Major clusters, which share the same PFGE pattern/serotype combination, were defined as those that included three or more pneumococcal isolates. In order to assess identity with global pneumococcal clones, at least one isolate of each cluster ($n = 42$) was analyzed by multilocus sequence typing (MLST), as described previously (29). Allele numbers and sequence types (ST) were assigned using the MLST web site (http://www.mlst.net).

**PCR amplification and DNA sequence determination.** *parE* and *parC* QRDRs were amplified by using the oligonucleotides parE398 (30) and parC152 (9). To amplify and sequence the *gyrA* QRDRs, the oligonucleotides gyrA44 and gyrA170 (30) were used. These PCR fragments were sequenced as previously described (17). To detect the presence of the *ant* gene, the oligonucleotides used in PCR amplifications were antUP and antDOWN (31).

**Statistical analysis.** The $\chi^2$ test or Fisher's exact test were used as appropriate. Two-sided *P* values of <0.05 were considered statistically significant.

## RESULTS

**Ciprofloxacin resistance and multidrug resistance.** The rate of CIP resistance in 2012 was 2.3% (83/3,621). Among these 83 CIP-resistant (Cip$^r$) isolates, 15 (18.1%) with MICs of 4 to 8 μg/ml were classified as low-level resistant (LL-Cip$^r$), while the remaining 68 (81.9%), with MICs of ≥16 μg/ml, were classified as high-level resistant (HL-Cip$^r$) (Table 1). Global Cip$^r$ rates remained stable across the three time periods studied (2002, 2006, and 2012), and no statistically significant variations were found in the rates of LL- and HL-Cip$^r$ isolates (Table 1). In addition, there was no difference between the three sets of results in relation to age groups except for a decrease in the prevalence of Cip$^r$ among pneumococci isolated from patients >64 years old (7.2% in 2002 versus 3.9% in 2012). In 2012, Cip$^r$ rates among pneumococci isolated from noninvasive disease (3.7%; 51/1,369) were higher than those for pneumococci isolated from invasive disease (1.4%; 32/2,252; $P < 0.001$), this being consistent with the previous two reports (17, 18).

**TABLE 2** Fluoroquinolone MICs of 83 isolates and amino acid changes in their DNA topoisomerase genes[a]

| No. of isolates | Amino acid substitution for: | | | | | | | | | MIC (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ParC | | | ParE | | | GyrA | | | | | |
| | D78 | S79 | D83 | K426 | D435 | E474 | G79 | S81 | E85 | CIP | LVX | MOX |
| 1 | — | — | — | — | — | — | — | — | — | 4 | 2 | 0.25 |
| 3 | — | F | — | — | — | — | — | — | — | 4–8 | 1–4 | 0.25–0.5 |
| 6 | — | Y | — | — | — | — | — | — | — | 4–8 | 1–2 | 0.12–8 |
| 3 | — | — | N | — | — | — | — | — | — | 4–8 | 1–4 | 0.25–0.5 |
| 1 | N | — | — | —‡ | —‡ | —‡ | —‡ | —‡ | —‡ | 8 | 2 | 0.5 |
| 1 | — | — | — | — | — | K | — | — | — | 8 | 2 | 1 |
| 1 | N | — | | — | — | — | — | F | — | 32 | 8 | 4 |
| 1 | — | F | — | — | — | — | A | — | — | 32 | 16 | 8 |
| 1 | — | F | — | — | — | — | — | A | — | 64 | 32 | 8 |
| 31 | — | F | — | — | — | — | — | F | — | 32–64 | 16–32 | 2–8 |
| 1 | — | F | — | — | — | — | — | Y | — | 32 | 16 | 2 |
| 1 | —‡ | Y | —‡ | —‡ | —‡ | —‡ | —‡ | F‡ | —‡ | 32 | 16 | 4 |
| 5 | — | Y | — | — | — | — | — | F | — | 32–64 | 16–32 | 2–4 |
| 1 | — | Y | — | — | — | — | — | V | — | 32 | 16 | 4 |
| 2 | — | Y | — | — | — | — | — | Y | — | 32 | 16 | 4 |
| 2 | — | F | — | — | — | — | — | — | K | 32 | 16 | 4 |
| 2 | — | — | Y | — | — | — | — | F | — | 32 | 16 | 2–4 |
| 1 | — | — | — | — | N | — | — | A | — | 64 | 32 | 1 |
| 1 | — | — | — | — | N | — | — | F | — | 32 | 16 | 2 |
| 1 | — | — | — | — | N | — | —‡ | F‡ | —‡ | 32 | 16 | 8 |
| 2 | — | F | N | — | — | — | — | F | — | 64 | 32 | 8 |
| 1 | — | F | Y | — | — | — | — | Y | — | 64 | 32 | 8 |
| 1 | — | Y | — | — | — | — | — | F | K | 64 | 32 | 1 |
| 1 | — | F | — | — | — | — | — | Y | K | 64 | 32 | 4 |
| 4 | — | F | — | — | — | — | — | F | K | 64 | 32 | 4–8 |
| 1 | — | Y | — | — | — | — | — | F | Q | 64 | 32 | 16 |
| 2 | — | F | — | — | N | — | — | F | — | 128 | 64 | 8 |
| 1 | — | Y | — | — | N | — | — | F | — | 128 | 64 | 4 |
| 1 | — | Y | — | N | — | — | — | F | — | 64 | 32 | 64 |
| 1 | — | — | N | — | — | K | — | Y | — | 32 | 16 | 2 |
| 1 | — | F | Y | N | — | — | — | V | | 128 | 64 | 8 |
| 1 | — | F | — | — | N | — | — | F | K | 128 | 64 | 64 |

[a] Only changes involved in resistance are shown. —, no change; ‡, the residue is located in a recombinant gene. Additional amino acid changes, not involved in resistance, were ParC N91D (the isolate with mosaic *parC* gene), ParE I460V (28 isolates), and GyrA S114G (the three isolates with mosaic *gyrA* genes). CIP, ciprofloxacin; LVX, levofloxacin; MOX, moxifloxacin.

Forty-eight (57.8%) Cip$^r$ pneumococci were considered multidrug resistant (MDR), defined as resistance to CIP plus at least two other antimicrobial groups. The MDR rate showed a slight increase from 2006 to 2012 (Table 1).

**Amino acid substitutions in QRDRs of pneumococcal isolates.** The *parC*, *parE*, and *gyrA* QRDRs of the 83 Cip$^r$ (MIC ≥ 4 μg/ml) isolates were characterized. In addition, 15 randomized isolates with CIP MICs of 2 μg/ml were analyzed, with their QRDRs showing susceptible sequences, in agreement with previous findings of our group (17, 18). QRDRs of *parE* and *parC* were amplified in a single PCR with the oligonucleotides parE398 and parC152. All isolates yielded 1.6-kb PCR products, with the exception of a recombinant strain, which yielded a bigger fragment (see below). Although most Cip$^r$ isolates (79/83) showed low nucleotide sequence variation (≤1%), four isolates exhibited high variation (>4%), suggesting a recombinant origin for these genes (see below). The different patterns of amino acid substitutions in the QRDRs of all Cip$^r$ pneumococci, as well as their MICs to FQs, are shown in Table 2. Among the 15 LL-Cip$^r$ isolates, all but 1 had mutations producing amino acid changes in topoisomerase IV subunits: 13 produced changes in ParC, and 1 did so in ParE. The remaining isolate, with a CIP MIC of 4 μg/ml, presented no changes in its QRDRs.

All HL-Cip$^r$ isolates had at least one amino acid change in topoisomerase IV genes, as well as mutations producing changes in the gyrase A subunit (Table 2). Among the 68 HL-Cip$^r$ isolates, 51 had double changes (70.6% at ParC and GyrA and 4.4% at ParE and GyrA), 15 had triple changes (either 1 or 2 changes at ParC and 1 or 2 changes at GyrA or 1 change each at ParC, ParE, and GyrA), and the remaining 2 isolates had four changes (one isolate had 2 ParC, 1 ParE, and 1 GyrA changes, while the other had 1 ParC, 1 ParE, and 2 GyrA changes). Mutations found were classical mutations involved in resistance, which have been previously found in clinical isolates and shown to be involved in resistance by genetic transformation (10, 12, 17, 18, 32–35).

Three isolates carried recombinant genes, one in *parE*, *parC*, and *gyrA*, one in *parE* and *gyrA*, and one in *gyrA*. These isolates probably acquired these genes from resistant *Streptococcus mitis* group isolates given the presence of ParC N91D in their ParC recombinant proteins and of GyrA S114G in their GyrA proteins (31). In addition, amplification of the isolate with the *parE* plus *parC* mosaic genes using oligonucleotides parE398 and parC152

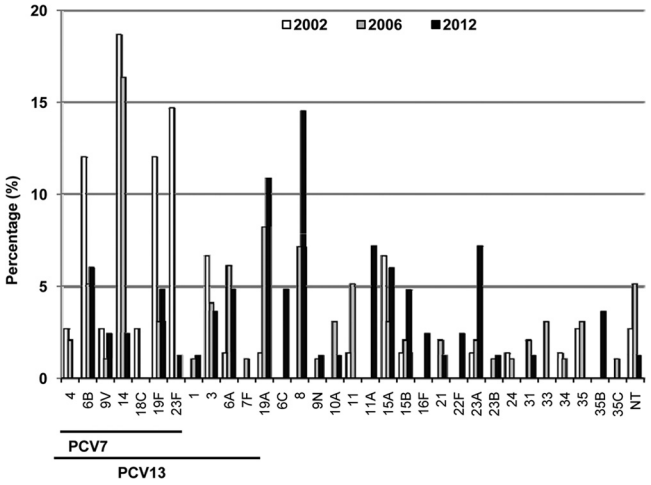


**FIG 1** Serotype distribution of ciprofloxacin-resistant pneumococci isolated in Spain in 2002, 2006, and 2012. A total of 75 isolates from 2002 (white columns), 98 from 2006 (gray columns), and 83 from 2012 (black columns) were compared. "PCV7" and "PCV13" indicate serotypes included in the respective conjugate pneumococcal vaccines.

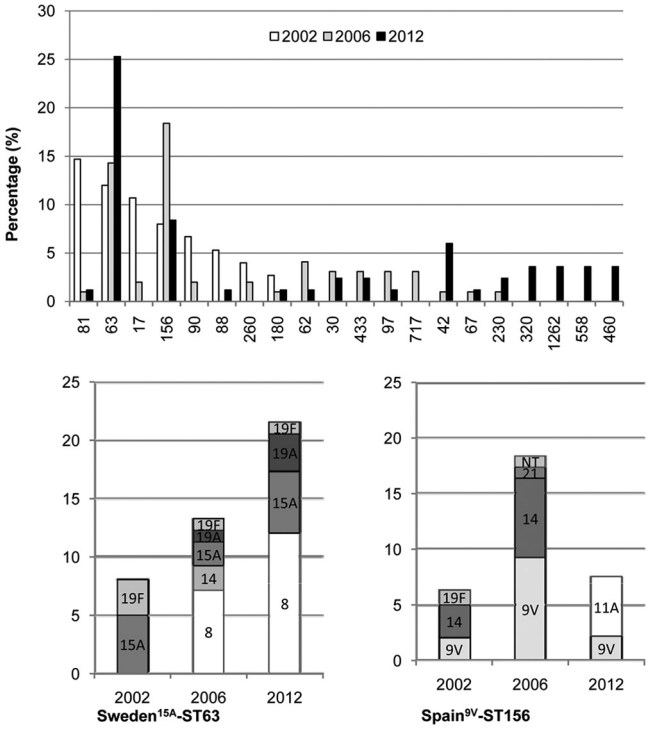


**FIG 2** Genotype of ciprofloxacin-resistant pneumococci isolated in Spain in 2002, 2006, and 2012 (top panel) or serotypes expressed in the indicated clones (bottom panels).

rendered a fragment of 3.5 kb, which is longer than the 1.6 kb observed in the remaining isolates. This characteristic is typical of *S. mitis* group isolates. In addition, an *ant* gene was detected by PCR amplification (31) in the intergenic *parE-parC* region of this isolate (data not shown).

**Dynamics of pneumococcal serotypes and genotypes.** A total of 24 different serotypes were detected among the Cip$^r$ pneumococci, but 6 of them accounted for 51.8% (43 of 83) of the isolates (Fig. 1): 8 (14.5%), 19A (10.8%), 11A (7.2%), 23A (7.2%), 6B (6.0%), and 15A (6.0%). A gradual decrease in PCV7 serotypes was found over the years from 2002 (65.3%) to 2006 (35.7%) to 2012 (16.9%), the most important concerning serotype 14 (Table 1). In Spain, PCV13 was licensed for adults in 2012, with the percentage of Cip$^r$ pneumococci belonging to PCV13 serotypes in that year being 37.3%, lower than the figure for 2006 (48.0%; $P = 0.176$). However, if one considers only the PCV13 serotypes not included in the PCV7 (1, 3, 5, 6A, 7F, and 19A), then a slight increase was observed (16.3% [16/98] in 2006, versus 20.5% [17/83] in 2012; $P = 0.563$); this increase was due mainly to the appearance and spread of serotype 19A, which was ranked second in 2012. It should also be noted that two non-PCV13 serotypes were detected in 21.7% of the overall Cip$^r$ episodes: serotype 8, which emerged in 2006 and whose frequency increased again in 2012 (Table 1), and serotype 11A, which showed a stepwise increase across the three sets of data.

Genetic relatedness among resistant isolates was determined primarily by PFGE in order to make comparisons with global clones, while representative isolates were further studied in terms of their allelic profiles by MLST ($n = 42/83$). Although 32 different PFGE clonal complexes (CCs) were detected among the Cip$^r$ isolates, 3 of them (CC63, CC156, and CC42) accounted for nearly 40% of isolates (Fig. 2). The CC63 clone, expressing serotypes 8, 15A, 19A, and 19F, was found in 25.3% (21/83) of Cip$^r$ isolates (Table 3); CC156, with serotypes 9V and 11A, was found in 8.4% (7/83), while CC42, with serotype 23A, accounted for 6.0% (5/83) (Fig. 2). In addition, there were four CCs, with three isolates each, which had not been detected in the previous studies: CC1662 with serotype 15B, CC558 with serotype 35B, CC320 with serotype 19A, and CC460 with serotype 6A.

The two most frequent CCs identified in 2012 (CC63 and CC156) had isolates with different serotypes, suggesting capsular switch events (Fig. 2). The CC63 clone showed a dramatic increase after 2002, and it was detected in 25.3% of Cip$^r$ pneumococci in 2012, associated mainly with serotype 8 (12 out of 21 isolates). Likewise, CC156 was maintained in 2012 and was associated with serotype 11A (5 out of 7 isolates).

## DISCUSSION

Current rates of fluoroquinolone resistance in Spain (2.3% for CIP) are similar to those reported previously in 2002 (2.6%) and 2006 (2.3%). The prevalence of FQ resistance has been directly correlated with the consumption of FQs, especially CIP (14, 25). Data from the Spanish Medicines Agency (http//agemed.es) indicate that CIP consumption in Spain has remained essentially stable since 2002, namely, at 1.1 defined daily dose per 1,000 inhabitants per day (DDD). Over the same period, MOX consumption showed only a slight variation, from 0.3 (2002) to 0.4 (2006) and back to 0.3 DDD (2012). However, LVX consumption has increased from 0.2 DDD in 2002 to 0.4 DDD in 2006 to 0.6 DDD in 2012.

In line with a previous study from Canada (36), this increase in FQ consumption has not led to increased FQ resistance rates. One explanation for this could be the greater efficacy of the new FQs, which may reduce selective pressure in relation to the pneumococcal QRDR mutations involved in FQ resistance (37). In addition, due to their toxicity, FQs are not used to treat children, and therefore they do not produce selective pressure on the pneumo-

**TABLE 3** Phenotypic characteristics and changes in QRDRs among 21 isolates of the CC63 clone found in 2012[a]

| Strain ID[b] | Serotype | Amino acid changes in QRDR of: | | | | | | No. of resistant mutations | MIC (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ParC | | ParE | | GyrA | | | | | |
| | | S79 | D83 | K426 | I460 | S81 | E85 | | CIP | LVX | MOX |
| 14 | 8 | **F** | — | — | V | **A** | — | 2 | 64 | 32 | 8 |
| 3–6, 19, 20, 96 | 8 | **F** | — | — | V | **F** | — | 2 | 64 | 32 | 4–8 |
| 47 | 8 | **Y** | — | — | V | **F** | — | 2 | 64 | 32 | 4 |
| 55 | 8 | **Y** | — | — | — | **F** | — | 2 | 64 | 32 | 2 |
| 24, 56 | 8 | **F** | **N** | — | V | **F** | — | 3 | 64 | 32 | 4 |
| 91 | 15A | — | **Y** | — | V | **F** | — | 2 | 32 | 16 | 2 |
| 53 | 15A | **Y** | — | — | V | **F** | — | 2 | 32 | 16 | 2 |
| 71 | 15A | **F** | — | — | V | **F** | — | 2 | 64 | 32 | 4 |
| 58 | 15A | **Y** | — | — | V | **F** | **Q** | 3 | 64 | 32 | 16 |
| 26 | 15A | **F** | **Y** | **N** | V | **V** | — | 4 | 128 | 64 | 8 |
| 81 | 19A | **Y** | — | — | V | — | — | 1 | 4 | 1 | 4 |
| 12 | 19A | **F** | — | — | V | **F** | — | 2 | 64 | 32 | 4 |
| 9 | 19A | **Y** | — | — | — | **F** | **K** | 3 | 64 | 32 | 8 |
| 51 | 19F | **F** | — | — | V | **F** | — | 2 | 64 | 32 | 2 |

[a] Changes involved in resistance are shown in bold type. —, no change. CIP, ciprofloxacin; LVX, levofloxacin; MOX, moxifloxacin.
[b] Strain identification no.

coccal population colonizing the nasopharynx of children (the main pneumococcal reservoir). In fact, in the present series, only two Cip$^r$ pneumococci were isolated from children (ages 1 and 3), and these cases were probably due to family cross-transmission.

The introduction of PCV7 in June 2001 and likely also that of PCV13 in June 2010 had an impact on the ecology of pneumococci causing disease in children and adults, but it has also substantially reduced the incidence of antibiotic resistance (2); in fact, the majority (65.3%) of Cip$^r$ isolates in 2002 belonged to serotypes included in PCV7, compared with only 35.7% of Cip$^r$ isolates in 2006 and 16.9% in 2012. In line with the changes found in the serotype distribution, the MDR rate also decreased in 2006 as a consequence of the decreased number of MDR clones associated with these PCV7 serotypes: CC81 (Spain$^{23F}$-ST81), CC90 (Spain$^{6B}$-ST90), CC17 (Spain$^{14}$-ST17), and CC88 of serotype 19F. However, although these clones have almost disappeared in 2012, MDR rates increased in this period ($P = 0.296$), mainly due to the increase and/or emergence of CCs associated with non-PCV7 MDR serotypes: CC63 (serotypes 8 and 15A), CC320 (serotype 19A), and CC42 (serotype 23A). These changes in clone and serotype distribution reflect changes in the pneumococcal population isolated from the nasopharynx of children, in which serotypes 15A, 15B, 19A, 6C, and 11A have increased in recent years (38, 39), and also in respiratory samples from acute exacerbations in COPD patients (40).

In comparison with the data for 2002 and 2006, the most notorious Cip$^r$ isolates in 2012 were those expressing serotypes 8 and 11A, not included in PCV13. All serotype 8 isolates were associated with genotype CC63, suggesting a capsular switching event. This clone expressing serotype 8 was first detected in the 2006 study (18), and it was disseminated in the Madrid area, mainly among HIV-positive patients, with one isolate showing FQ resistance and a ParC S79F amino acid substitution (26, 41). In the present series, eight CC63-serotype 8 isolates were also isolated from patients at hospitals in Madrid, a fact that could explain the frequency of this serotype among Cip$^r$ pneumococci in 2012.

CC63 was the most frequently detected CC (21 of 83 Cip$^r$ isolates) in this study and expressed four serotypes (8, 15A, 19A, and 19F). Of these, only 19A and 19F are included in PCV13. These 21 isolates had either one (1 isolate), two (15 isolates), three (4 isolates), or four (1 isolate) mutations at *parC*, *parE*, or *gyrA*. Heterogeneity was observed in terms of both the amino acid (S79 or D83) affected at ParC and the change producing resistance. There was also heterogeneity in GyrA mutations, which were found at either S81 or E85. These results suggest that although some of the isolates could have a clonal origin, the majority of these Cip$^r$ isolates are likely to be the result of spontaneous mutations in a CC63 isolate, which became predominant among Cip$^r$ pneumococci in 2012. In agreement with this, the CC63 genotype ranked first (9.1%) among 206 noninvasive pneumococci collected from chronic obstructive pulmonary disease patients during 2009 to 2012, and 5/18 (27.8%) of them showed resistance to FQs (42). In contrast, although we have no data about the genotypes of all pneumococci sent to the Reference Laboratory in 2012, data from Barcelona reveal that the overall frequency of CC63 among invasive isolates was low: 3.3% (37/1121) in adults in the years 1997 to 2008 (6) and 1.5% (3/198) in children in the 1997-to-2006 period (42).

Regarding serotype 11A, the two previous studies reported that pneumococci expressing this serotype were related to the ST62 clone. In the present series, however, five of six Cip$^r$ pneumococci expressing serotype 11A belonged to genotype CC156, suggesting another capsular switching event. This is a cause of concern because it allows the persistence of a well-established clone that usually expresses PCV7 serotypes (9V and 14) through a vaccine escape phenomenon.

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