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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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ABSTRACT

In Spain, rates of ciprofloxacin resistance in pneumococci were low during the last decade (2.6% in 2002; 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 4-8 μg/ml) and 68 a high level (MIC of 16-128 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, six 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 PCV7 serotypes was observed from 2006 (35.7%) to 2012 (16.9%), especially of serotype 14 (from 16.3% to 2.1%; $P<0.001$). In comparison with 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, 19A and 19F; CC320 (with serotype 19A); 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 pre-existing 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 invasive disease incidence due to PCV7 serotypes was also observed (5). However, shortly after PCV7 introduction an emergence of non-vaccine 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 susceptibility to FQs varies across bacterial species (8). Isolates of *S. pneumoniae* resistant to FQs have been shown to present mutations at specific regions (quinolone-resistant determining regions, QRDRs) of the topoisomerase IV (*parC*, *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 it 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 when compared with those of LVX and MOX are due to

isolates with first-step QRDRs 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≥ 4 μg/ml we have detected first-step mutations in isolates susceptible to LVX by the CLSI criteria (LVX MIC 1-2 μg/ml). In addition, among isolates with 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, 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 revealed 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 from children. In terms of their origin, 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 MIC ≥ 4 μg/ml were confirmed by E-test and broth microdilution methods, according to the Clinical and Laboratory Standards Institute guidelines (27). *S. pneumoniae* ATCC 49619 was included as 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 *Sma*I or *Apa*I and fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad). PFGE patterns were visually compared with 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 multi-locus 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, 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 χ^2 test or Fisher's exact test were used as appropriate. Two-sided *P* values < 0.05 were considered statistically significant.

RESULTS

Ciprofloxacin resistance and multi-drug resistance. The rate of CIP resistance in 2012 was 2.3% (83/3,621). Among these 83 CIP-resistant (CipR) isolates, 15 (18.1%) with MICs of 4-8 μg/ml were classified as low-level resistant (LL-CipR), while the remaining 68 (81.9%) with MICs of ≥16 μg/ml were classified as high-level resistant (HL-CipR) (Table 1). Global CipR 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-CipR 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 CipR among pneumococci isolated from patients >64 years old (7.2% in 2002 *vs.* 3.9% in 2012). In 2012, CipR rates among pneumococci isolated from non-invasive disease (3.7%, 51/1369)

were higher than those for pneumococci isolated from invasive disease (1.4%, 32/2252; *P<0.001*), this being consistent with the previous two reports (17, 18).

Forty-eight (57.8%) CipR 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 the QRDRs of pneumococcal isolates. The *parC*, *parE*, and *gyrA* QRDRs of the 83 CipR (MIC ≥4 μg/ml) isolates were characterized. In addition, 15 randomized isolates with CIP MICs = 2μg/ml were analysed, their QRDRs showing susceptible sequences, in agreement with previous studies of our group (17, 18). QRDRs of *parE* and *parC* were amplified in a single PCR reaction with oligonucleotides parE398 and parC152. All isolates yielded 1.6 Kb PCR reaction products, with the exception of a recombinant strain, which yielded a bigger fragment (see below). Although most CipR isolates (79/83) showed low nucleotide sequence variations (≤1%), four isolates exhibited high variations (>4%), suggesting a recombinant origin for these genes (see below). The different patterns of amino acid substitutions in the QRDRs of all CipR pneumococci, as well as their MICs to FQs, are shown in Table 2. Among the 15 LL-CipR isolates, all but one had mutations producing amino acid changes in topoisomerase IV subunits: 13 produced changes in ParC and one did so in ParE. The remaining isolate with a CIP MIC of 4 μg/ ml presented no changes in its QRDRs.

All HL-CipR 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-CipR isolates, 51 had double changes (70.6% at ParC + GyrA; 4.4% at ParE + GyrA), 15 had triple changes (either 1 or 2 changes at ParC + 1 or 2 changes at GyrA, or 1 change each at

159 ParC, ParE, and GyrA), and the remaining two isolates had four changes (one isolate had 2
160 ParC + 1 ParE + 1 GyrA changes, while the other had 1 ParC + 1 ParE + 2 GyrA changes).
161 Mutations found were classical mutations involved in resistance, which have been previously
162 found in clinical isolates and shown to be involved in resistance by genetic transformation
163 (10, 12, 17, 18, 32-35).

164 Three isolates carried recombinant genes, one in *parE* + *parC* + *gyrA,* one in *parE* +
165 *gyrA,* and one in *gyrA*. These isolates probably acquired these genes from *Streptococcus*
166 *mitis* group resistant isolates, given the presence of ParC N91D in their ParC recombinant
167 proteins and of GyrA S114G in their GyrA proteins (31). In addition, amplification of the
168 isolate with *parE* + *parC* mosaic genes using oligonucleotides parE398 and parC152
169 rendered a fragment of 3.5 kb, which is longer than the 1.6 kb observed in the remaining
170 isolates. This characteristic is typical of *S. mitis* group isolates. In addition, an *ant* gene was
171 detected by PCR amplification (31) in the intergenic *parE-parC* region of this isolate (data
172 not shown).

173 **Dynamics of pneumococcal serotypes and genotypes.** A total of 24 different
174 serotypes were detected among the CipR pneumococci, but six of them accounted for
175 51.8% (43 of 83) of the isolates (Fig. 1): 8 (14.5%), 19A (10.8%), 11A (7.2%), 23A
176 (7.2%), 6B (6.0%), and 15A (6.0%). A gradual decrease in PCV7 serotypes was found over
177 the years from 2002 (65.3%) to 2006 (35.7%) to 2012 (16.9%), the most important
178 concerning serotype 14 (Table 1). In Spain, PCV13 was licensed for adults in 2012, with
179 the percentage of CipR pneumococci belonging to PCV13 serotypes in that year being
180 37.3%, lower than the figure for 2006 (48.0%; P=0.176). However, if one considers only
181 the PCV13 serotypes not included in the PCV7 (1, 3, 5, 6A, 7F, and 19A), then a slight
182 increase was observed [16.3% (16/98) in 2006 *vs.* 20.5% (17/83) in 2012, *P=0.563*]; this

183 increase was mainly due to the appearance and spread of serotype 19A, which was ranked
184 second in 2012. It should also be noted that two non-PCV13 serotypes were detected in
185 21.7% of the overall CipR episodes: serotype 8, which emerged in 2006 and which
186 frequency increased again in 2012 (Table 1), and serotype 11A, which showed a stepwise
187 increase across the three sets of data.

188 Genetic relatedness among resistant isolates was determined primarily by PFGE in
189 order to make comparisons with global clones, while representative isolates were further
190 studied in terms of their allelic profiles by MLST (n=42/83). Although 32 different PFGE
191 clonal complexes (CCs) were detected among the CipR isolates, three of them (CC63,
192 CC156, and CC42) accounted for nearly 40% of isolates (Fig. 2B). The CC63 clone,
193 expressing serotypes 8, 15A, 19A and 19F, was found in 25.3% (21/83) of CipR isolates
194 (Table 3); CC156, with serotypes 9V and 11A, was found in 8.4% (7/83), while CC42, with
195 serotype 23A, accounted for 6.0% (5/83) (Fig. 1B). In addition, there were four CCs, with
196 three isolates each, which had not been detected in the previous studies: CC1662 with
197 serotype 15B, CC558 with serotype 35B, CC320 with serotype 19A, and CC460 with
198 serotype 6A.

199 The two most frequent CCs in 2012 (CC63 and CC156) had isolates with different
200 serotypes, suggesting capsular switch events (Fig 2). The CC63 clone showed a dramatic
201 increase since 2002, and it was detected in 25.3% of CipR pneumococci in 2012, mainly
202 associated with serotype 8 (12 out of 21 isolates). Likewise, CC156 was maintained in 2012
203 and was associated with serotype 11A (5 out of 7 isolates).

204

205 **DISCUSSION**

206 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 DDD (defined daily doses per 1000 inhabitants per day). 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, FQs are not, due to their toxicity, used to treat children, and, therefore, they do not produce selective pressure on the pneumococcal population colonizing the nasopharynx of children (the main pneumococcal reservoir). In fact, in the present series only two CipR pneumococci were isolated from children (aged one and three), 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 CipR isolates in 2002 belonged to serotypes included in PCV7, compared with only 35.7% of CipR 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}-

231 ST81), CC90 (Spain^{6B}-ST90), CC17 (Spain^{14}-ST17), and CC88 of serotype 19F. However,
232 although these clones have almost disappeared in 2012, MDR rates increased in this period
233 (P=0.296, mainly due to the increase and/or emergence of CCs associated with non-PCV7
234 MDR serotypes: CC63 (serotypes 8 and 15A), CC320 (serotype 19A), and CC42 (serotype
235 23A). These changes in clone and serotype distribution reflect changes in the
236 pneumococcal population isolated from the nasopharynx of children, in which serotypes
237 15A, 15B, 19A, 6C, and 11A have increased in recent years (38, 39); and also in respiratory
238 samples from acute exacerbations in COPD patients (40).

239 In comparison with the data for 2002 and 2006, the most notorious CipR isolates in
240 2012 were those expressing serotypes 8 and 11A, not included in PCV13. All serotype 8
241 isolates were associated with genotype CC63, suggesting a capsular switching event. This
242 clone expressing serotype 8 was first detected in the 2006 study (18), and it was
243 disseminated in Madrid area, mainly among positive HIV patients, one which showed FQ
244 resistance and a ParC S79F amino acid substitution (26, 41). In the present series, eight of
245 CC63-serotype 8 isolates were also isolated from patients attended at Hospitals of Madrid,
246 a fact that could explain the frequency of this serotype among CipR pneumococci in 2012.

247 CC63 was the most frequently detected (21 of 83 CipR isolates) in this study, and
248 expressed four serotypes (8, 15A, 19A, and 19F). Of them, only 19A and 19F are included
249 in PCV13**.** These 21 isolates had either one (1 isolate), two (15 isolates), three (4 isolates),
250 or four (1 isolate) mutations at *parC*, *parE*, or *gyrA*. Heterogeneity was observed in terms
251 of both the amino acid (S79 or D83) affected at ParC and the change producing resistance.
252 There was also heterogeneity in GyrA mutations, which were found either at S81 or E85.
253 These results suggest that although some of the isolates could have a clonal origin, the
254 majority of these CipR isolates are likely to be the result of spontaneous mutations in a

CC63 isolate, which become predominant among CipR pneumococci of year 2012. In agreement with this, CC63 genotype ranked first (9.1%) among 206 non-invasive pneumococci collected from chronic obstructive pulmonary disease patients during 2009-2012 and 5/18 (27.8%) of them showed resistance to FQs (42). In contrast, although we have not data about the genotypes of all pneumococci sent to the Reference Laboratory in 2012, data from Barcelona reveals that the overall frequency of CC63 among invasive isolates was low: 3.3% (37/1121) in adults in years 1997-2008 (6) and 1.5% (3/198) in children in the 1997-2006 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 CipR 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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FIGURE LEGENDS

FIGURE 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 (grey columns), and 83 from 2012 (black columns) were compared. PCV7 and PCV13 indicate serotypes included in the respective conjugate pneumococcal vaccines.

FIGURE 2. Genotype of ciprofloxacin-resistant pneumococci isolated in Spain in 2002, 2006, and 2012 (A), and serotypes expressed in the indicated clones (B).