Polyclonal Population Structure of mef and mef plus erm(B) in *Streptococcus pneumoniae* isolates in Spain

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The population structure (serotype, PFGE and MLST) of 45 mef-positive isolates were studied. They were selected among all erythromycin-resistant isolates (n=244) obtained within a collection of 712 isolates recovered from different Spanish geographic locations in the 1999-2003 pre-vaccination period. Overall resistance (CLSI criteria) rates among the 45 mef-positive isolates were: penicillin G, 82.2%; cefotaxime, 22.2%; clindamycin, 62.2%; and tetracycline, 68.8% (mainly in erm(B) plus mef(E), p<0.001). No levofloxacin or telithromycin resistance was found. Macrolide resistance phenotypes (disk diffusion approximation test) were: M, 37.7% [all but one due to mef(E)] and cMLS\textsubscript{B}, 62.2% [all mef(E) plus erm(B)]. Serotype 14 (22.2%), 6B (17.7%), 19A (13.3%) and 19F (11.1%) were predominant. Twenty-five different DNA patterns (PFGE) were observed. Our mef-positive isolates were grouped (eBURST analysis) into 4 clonal complexes (n=18) and 19 singleton clones (n=27). With the exception of Spain\textsuperscript{9V}-3, all clonal complexes (CC6B, Spain\textsuperscript{6B}-2 and Sweden\textsuperscript{15A}-25) and 73.6% of singleton clones carried both erm(B) plus mef genes. The international multi-resistant clones Spain\textsuperscript{23F}-1 and Poland\textsuperscript{6B}-20 were represented as singleton clones. A high proportion of mef-positive S. pneumoniae isolates presented the erm(B) gene, all expressing the cMLS\textsubscript{B} phenotype. A polyclonal population structure was demonstrated within our Spanish mef-positive S. pneumoniae isolates with few clonal complexes over-represented within this collection.
Macrolide resistance among *S. pneumoniae* clinical isolates has risen to prominence. It is even higher than that of penicillin, particularly in Spain and in some other European countries such as France, Poland, Greece and Portugal (17,22). In most European countries, erythromycin resistance is mainly due to the presence of the *erm*(B) gene. This gene encodes an rRNA-methylase responsible for the MLS\textsubscript{B} phenotype and is associated with conjugative transposons. Clonal dispersion of *erm*(B) positive *S. pneumoniae* isolates as well as horizontal transfer of transposable elements carrying this determinant, have been associated with the increase of erythromycin resistance in this organism (4,12). Erythromycin resistance may also be associated with the expression of efflux pumps encoded by *mef* genes that endowed the M phenotype (7). This phenotype has been traditionally more prevalent in North America than in Europe (7,12).

During the last years, the presence of both *erm*(B) and *mef*(E) genes in *S. pneumoniae* clinical isolates has been increasingly recognized, but they are more prevalent in Asian countries than in Europe or in the United States (10, 11). Despite the interest of this association for the evolution of macrolide and ketolide resistance, very few studies have investigated the population biology within a collection of isolates recovered along different time periods and/or geographic origins (13, 26). In Spain, population structure studies have shown that resistance in *S. pneumoniae* international clones such as Spain\textsuperscript{9V}-3 and England\textsuperscript{14-9} are mainly associated with those endowed with the M phenotype (1,2) whereas Spain\textsuperscript{23F-1} and Spain\textsuperscript{6B-2} are associated with isolates endowed with the MLS\textsubscript{B} phenotype (14). This type of analysis in *S. pneumoniae* isolates with both *erm*(B) and *mef* genes has not been reported. In the present study, the population structure and clonal relatedness of 45 *mef*-positive isolates (62.2% of them with both *erm*(B) and *mef* genes) identified within a collection of 712 *S. pneumoniae* isolates recovered at different Spanish hospitals during 1999-2003 was fully.
investigated. Moreover, the corresponding resistance phenotypes and antibiotic susceptibility patterns were also studied.

MATERIAL AND METHODS

**Bacterial isolates.** Seven hundred and twelve *S. pneumoniae* clinical isolates (244 isolates non-susceptible to erythromycin) recovered during the pre-vaccination period (in Spain the use of conjugate heptavalent vaccine was accepted in 2002, but was rarely used until 2004), were studied. Isolates were prospectively collected in 14 Spanish hospitals representing 14 different geographic areas during fall and winter seasons of 1999 through 2003. Among these strains, a total of 45 *mef*-positive isolates were detected and all of them selected for further studies. The origin of these 45 isolates is included in table 1. 26.6% (12 out of 45) of these isolates were of paediatric origin.

**Susceptibility testing and erythromycin resistance phenotypes.** MICs of penicillin, cefotaxime, erythromycin, clindamycin, telithromycin, tetracycline, and levofloxacin were performed by broth microdilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines (3). Incubation was performed at 35°C in ambient air. *S. pneumoniae* ATCC 49619 was used as reference strain in each run. Breakpoints were those established by the CLSI guidelines (3). The phenotypic detection of *erm*-induction was performed using the macrolide (erythromycin)-clindamycin disk diffusion approximation test as previously described (17).

**Detection of erythromycin resistance genes.** A real time PCR approach was carried out for *erm* and *mef* genes detection among erythromycin-resistant isolates using conditions previously described (20). A subsequent scheme of multiplex-PCR was followed to differentiate between *mef*(A) and *mef*(E) genes. Specific primers designed for this purpose were mefAF 5’-AATACAATTTGAAACTT- 3’, mef EF 5’-
AAGGAGTTGTGGTTCTGA- 3’, and the reverse primer for both \textit{mef}(A) and \textit{mef}(E) genes \textit{mef} RF 5’-AATCGGTAAATCATTGG- 3’. Expected sizes of PCR products were: 1,080 kb for \textit{mef}(A) and 480 kb for \textit{mef}(E), respectively. The PCR amplification mixture of 25 µL contained: 15 mM Tris-HCl, 50 mM KCl (pH 8.0), and 25 mM MgCl₂, 100 µM of each nucleotide, 0.15 pmol of the two forward primers \textit{mef}AF and \textit{mef}EF, 0.3 pmol of the reverse primer \textit{mef}RF, 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) and 1 µl of genomic DNA. PCR conditions (PTC-100 thermocycler, MJ Research Inc, Watertown, Mass) comprised an initial denaturation step at 94°C for 12 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and elongation at 72°C for 1 min. After amplification cycles, a final elongation step of 10 min at 72°C was included.

**Population structure.** Serotyping was performed with the Neufeld Quellung reaction using antisera provided by the Statens Seruminstitut (Copenhagen, Denmark). Pulsed field gel electrophoresis (PFGE) was performed as previously described by del Campo et al. (6). Briefly, chromosomal DNA was prepared following the standard Gram-positive protocol with some modifications (6). DNA restriction was made with \textit{Sma}I endonuclease (Amersham Biosciences Europe GmbH, Freiburg, Germany). The electrophoresis was performed in CHEF DR-III equipment (Bio-Rad, Birmingham, UK) for 23h at 14°C, and the following settings were applied at 6 V/cm; 1-30 seconds. PFGE patterns were compared with those of clones established by the Pneumococcal Molecular Epidemiology Network (16).

Multilocus sequence typing (MLST) scheme for \textit{S. pneumoniae} was carried out as previously described (9). Clusters of related sequence types (STs) were grouped into clonal complexes (CCs) using eBURST (http://www.mlst.net). STs were clustered with the BioNumerics software (version 4.0; Applied Maths, Sint-Martens-Latem, Belgium)
using a categorical coefficient and a graphing method called minimum spanning tree (MST) as previously described (24).

**Statistical analysis.** Statistical associations were analyzed using chi-square test. Statistical significance was considered when two tailed p value was lower than 0.05.

**RESULTS**

**Macrolide resistance genes, phenotypes and susceptibility patterns.** Within the entire *S. pneumoniae* population (n=712), 244 isolates were non-susceptible to erythromycin. Among them, 80.7% carried the *erm*(B) gene as a sole genetic determinant affecting this compound and showed the MLS\(_B\) phenotype. In addition, 45 (18.4%) of the erythromycin resistant isolates carried a *mef*-type gene, and of these 44 isolates had the *mef*(E) gene and the remaining one the *mef*(A) gene. It is of note that 28 out of 45 *mef* positive isolates also carried the *erm*(B) gene (62.2%). In two isolates displaying erythromycin and clindamycin MIC values of 0.5 mg/L neither *erm*(B) gene nor the *mef* gene was detected. The MLS\(_B\) constitutive phenotype was observed in all 28 isolates carrying both *mef* and *erm*(B) genes, whereas the M phenotype was detected in the remaining 17 isolates that carried one of the *mef* gene alone. No temporal or local geographic association among *mef* or *mef* plus *erm*(B) positive isolates was observed (data not shown).

Overall penicillin G, cefotaxime, tetracycline and clindamycin resistance rates among all 45 *mef*-positive isolates were 82.2% (35.5% plus 46.7%); 22.2% (17.7% plus 4.4%); 68.8% (13.3% plus 55.5%) and 62.2% (0% plus 62.2%), respectively. Neither telithromycin resistance nor levofloxacin resistance was found (MIC ranges, 0.03-1 mg/L and 0.25-2 mg/L, respectively).
**Population structure in S. pneumoniae mef positive isolates.** The serotype distribution among the 45 mef positive isolates was as follows: 14, 22.2%; 6B, 17.7%; 19A, 13.3%; 19F, 11.1%; 11A, 6.6%; 9V, 6.6%; 15A, 6.6%; 23F, 4.4% and others, 11.1%. With a single exception, all isolates belonging to serotype 14 (n=10) harbored the mef gene alone. Within the mef(E) positive isolates, 6 different PFGE patterns were found, 58.8% of them belonging to the Spain9V-3 clone. Within the erm(B) plus mef(E) positive isolates, 19 different PFGE patterns were found with Sweden15A-25, Spain6B-2, Spain23F-1, Poland6B-20 and NorwayNT-42 clones represented. The eBURST analysis of the MLST-typing results showed that our mef-positive isolates were grouped into 4 clonal complexes and 19 singleton clones (Fig. 1). Serotypes, PFGE distribution according to e-BURST analysis and the corresponding STs are shown in table 1.

The clonal complexes grouped 18 of the mef positive isolates, 10 of them carrying the mefE gene as sole resistance determinant and belonging to the Spain9V-3 international clone, and 8 isolates carrying both erm(B) and mef(E) genes. These latter isolates were grouped into the Spain6B-2 international clone (n=3), the Sweden15A-25 (n=2) and the clonal complex 6B which includes two different STs (ST135 and ST1638, a double locus variant of ST135) (n=3).

Twenty-seven isolates were grouped into singleton clones which included some international resistant clones such as Spain23F-1 (two isolates harboring both erm(B) and mef(E) genes), Poland6B-20 and NorwayNT-42 (one isolate each carrying both erm(B) and mef(E) genes) and the England14-9 represented by the only isolate that carried the mef(A) gene detected in this study. With the exception of tetracycline (MIC 4 mg/L), this isolate was susceptible to all antibiotics tested. ST276 (single locus variant of Denmark14-32), ST549 and ST62 grouped more than one isolate each (4, 3 and 3
isolates, respectively) while other STs were each represented by a single isolate. Relationship between ST and macrolide-resistance genes is shown in table 1.

Serotype distribution according to eBURST analysis showed that despite the fact that strains of some serotypes belonged to specific international clones such as 6B, 14, 19A and 15A, these serotypes were also found among the isolates grouped as singleton clones. Serotype distribution according to eBURST analysis is shown in table 1.

Co-resistance with penicillin was found (MIC range 0.12-4 mg/L) among the isolates grouped into clonal complexes. The only isolate intermediate for cefotaxime (MIC 4 mg/L) belonged to the CC-Spain\textsuperscript{9V}-3. This isolate also showed increased MIC values for levofloxacin (2 mg/L). This was also found among the CC-Spain\textsuperscript{6B}-2 and CC-6B. Only 8 isolates (all of them belonging to CCSR\textsuperscript{9V}-3) were susceptible to tetracycline. The susceptibility pattern among the clonal complexes is shown in table 2.

Some differences among the isolates grouped as singleton clones were observed between the \textit{mef}-positive isolates and the isolates containing both \textit{erm}(B) and \textit{mef}(E).

Co-resistance with penicillin G was more prevalent among the \textit{erm}(B) plus \textit{mef}(E) isolates (89.2%) than among the \textit{mef}(E)/\textit{mef}(A)-positive (70.5%) isolates. The same trend, but with high statistical significance (P <0.001), was observed for tetracycline resistance among the dual \textit{erm}(B) plus \textit{mef}(E) isolates (92.8%) when compared with \textit{mef}(E)/\textit{mef}(A)-positive (29.4%) isolates. Cefotaxime resistance was similar between both groups. The susceptibility pattern of the singleton clones according to the macrolide resistance genotype is shown in table 3.
DISCUSSION

Typing studies are useful to understand the epidemiology and spread of resistant bacteria as well as to establish control protocols against epidemics (9). For S. pneumoniae, capsular typing is commonly used in clinical laboratories and has demonstrated that particular serotypes such as 23F and 6B are related to the worldwide spread of penicillin G resistant isolates (5, 20). The PFGE is useful to observe recent changes and MLST is valuable to discriminate the variations that slowly accumulate among the bacterial population (9). These techniques have been used in our study in all 45 erythromycin-resistant S. pneumoniae isolates carrying mef genes recovered during a prospective study in different Spanish geographic areas. With the aid of the MLST technique, mef positive isolates were demonstrated to be polyclonal in our Spanish S. pneumoniae isolates with few clonal complexes over-represented within the studied collection.

Resistance to macrolides in S. pneumoniae dramatically increased in Spain during the 1999-2003 pre-vaccination period (17, 22). In our collection, 34.3% of isolates were resistant to erythromycin, mainly due to the presence of the erm(B) gene. During the last years an increase in the prevalence of efflux mechanisms (M phenotype) has been observed (13, 20). Within the macrolide resistant isolates of our collection, the M phenotype was found in 6.9% of isolates and all of them carried a mef gene. However, the presence of this gene was even higher since 11.5% (28 out of 244 isolates) of the isolates with constitutive MLSB phenotype concomitantly presented the mef(E) gene. This figure is higher than that previously reported in other studies in Spain in which mef isolates did not have the erm(B) gene (2). Such an increasing association between mef(E) and erm(B) genes was unexpected in a country with a high incidence of S. pneumoniae isolates harbouring erm(B) gene. It is indeed difficult to understand the
possible selective advantage of these isolates harbouring both determinants, as \textit{erm}(B) alone provides higher MICs than those apparently needed to resist the action of macrolides. In other countries, the increased prevalence of isolates carrying both determinants has been related to the spread of specific clones such Taiwan$^{19F}$-14 and members of a specific clonal complex CC271, including ST271, ST236 and ST320 (8,10). It is of note that the ST271 was also found in our collection but as a singleton clone not related to the other STs (Fig. 1). In our study, the presence of isolates with both \textit{erm}(B) and \textit{mef}(E) genes was associated with three different clonal complexes and fourteen singleton clones (Table 1). These results demonstrated the non-clonal nature of the studied population and that horizontal gene transfer processes might have occurred as well as the selection of resistant \textit{S. pneumoniae} isolates harbouring \textit{mef}(E) plus \textit{erm}(B) genes.

In addition, most of the \textit{erm}(B) plus \textit{mef}(E) isolates were also resistant to penicillin, a fact that has also been found in geographic areas where this dual genotype is prevalent (10). On the other hand, tetracycline resistance was significant among the \textit{erm}(B) plus \textit{mef}(E) positive isolates. This fact could be related to the presence of conjugative transposons, like Tn2010, recently described in these isolates (4, 8). The participation of this trait in the maintenance of these isolates should be investigated.

Within the studied population with both \textit{erm}(B) and \textit{mef} determinants we were able to identify multi-resistant international clones (www.sph.emory.edu/PMEN) circulating in Spain (2, 14, 18), including Spain$^{6B}$-2, Sweden$^{15A}$-25 among the clonal complexes and Spain$^{23F}$-1, Poland $^{6B}$-20 and Norway$^{NT}$-42 as singleton clones. Both the Spain$^{6B}$-2 and Sweden$^{15A}$-25 clones have been shown to be over-represented in \textit{S. pneumoniae} isolates highly resistant to penicillin or with an MLS$_B$ phenotype (2, 14). Among the singleton clones, the Spain$^{23F}$-1 was scarcely represented in our collection (two isolates
of serotypes 19A and 23F), as were the Poland\textsuperscript{6B}-20 and Norway\textsuperscript{NT}-42 when compared with other studies (2,14). Moreover, we did not find the Spain\textsuperscript{14}-5, which also carries both genes, as in some specific Spanish geographic areas over a 22 year period (15,19). Among the isolates with the \textit{mef} gene as sole resistance determinant, only one isolate had the \textit{mef}(A) subclass. This isolate belonged to the international resistant clone England\textsuperscript{14}-9 also described in other European countries (1). The population of the \textit{mef}(E) positive isolates was structured into four singleton clones and a clonal complex belonging to the Spain\textsuperscript{9V}-3 international resistance clone with capsular switching into serotype 14 as previously described by Ardanuy et al. (1) and also into serotype 19A as has been observed in other studies (25). Only resistance of 14.2\% to penicillin was found among the \textit{mef}(E)-singleton clones, and MIC range for penicillin within the CC-Spain\textsuperscript{9V}-3 was 0.12-4 mg/L in agreement with other Spanish studies (1).

In conclusion, macrolide resistance in our Spanish \textit{S. pneumoniae} collection was high (34.3\%). An increase in the presence of the \textit{mef} gene could be noticed when compared with other studies, but this was not associated with the M phenotype. Most of the \textit{mef}-positive isolates also harboured the \textit{erm}(B) gene (62.2\% of the \textit{mef}-positive isolates) all of them showing the \textit{CMLS}\textsubscript{B} phenotype. Among the isolates showing the M phenotype only one carried the \textit{mef}(A) subclass, belonging to the England\textsuperscript{14}-9 as previously described in Europe (1). Population structure analysis showed that the \textit{mef}-positive isolates are grouped in different clones and clonal complexes and not only in a few clones as previously described in other geographic areas (1, 14).
Acknowledgements

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References


antimicrobial-resistant Spanish clones of *Streptococcus pneumoniae* (Spain23F-1, Spain68-2, Spain9V-3, and Spain14-5). Microb. Drug Resist. 9:133-137.


TABLE 1. Typing characteristics of 45 *mef* positive *Streptococcus pneumoniae* isolates recovered in Spain

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<thead>
<tr>
<th>Gene</th>
<th>MLST type</th>
<th>PFGE type or subtype</th>
<th>Serotype</th>
<th>Year of isolation</th>
<th>Origin (no. of isolates)</th>
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<td>Spain&lt;sup&gt;9V&lt;/sup&gt;-3</td>
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<td>Sputum (n=5), Ear (n=1), Blood (n=1)</td>
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<td>S 19F-ST271</td>
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<tr>
<td>$erm(B)+mef(E)$</td>
<td>ST 344 (n=1)</td>
<td>S Norway$^NT$-42</td>
<td>9V</td>
<td>2000-2001</td>
<td>Sputum (n=1)</td>
</tr>
<tr>
<td>$erm(B)+mef(E)$</td>
<td>ST 1486 (n=1)</td>
<td>S 6B-ST1486</td>
<td>6B</td>
<td>1999-2000</td>
<td>Sputum (n=1)</td>
</tr>
<tr>
<td>$erm(B)+mef(E)$</td>
<td>ST 1577 (n=1)</td>
<td>S 15C-ST1577</td>
<td>15C</td>
<td>2002-2003</td>
<td>Sputum (n=1)</td>
</tr>
<tr>
<td>$erm(B)+mef(E)$</td>
<td>ST 2639 (n=1)</td>
<td>S 10A-ST2639</td>
<td>10A</td>
<td>2002-2003</td>
<td>Bronchial aspirate (n=1)</td>
</tr>
<tr>
<td>$erm(B)+mef(E)$</td>
<td>ST 2820 (n=1)</td>
<td>S 12F-ST2820</td>
<td>12F</td>
<td>2002-2003</td>
<td>Sputum (n=1)</td>
</tr>
</tbody>
</table>
### TABLE 2. Susceptibility pattern of isolates belonging to different clonal complexes

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Spain&lt;sup&gt;9V&lt;/sup&gt;-3</th>
<th>Sweden&lt;sup&gt;13A&lt;/sup&gt;-25</th>
<th>Spain6B</th>
<th>CC-6B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mef(E) (n=10)</td>
<td>erm(B)+mef(E) (n=2)</td>
<td>erm(B)+mef(E) (n=3)</td>
<td>ermB(B)+mef(E) (n=3)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.5-16*</td>
<td>≥64</td>
<td>≥64</td>
<td>32-&gt;64</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.03-0.25</td>
<td>≥64</td>
<td>≥64</td>
<td>32-&gt;64</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>0.05-0.1</td>
<td>0.008-0.03</td>
<td>0.08-0.5</td>
<td>&lt;0.01-0.06</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5-4</td>
<td>≥64</td>
<td>≥64</td>
<td>4-&gt;64</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.12-4</td>
<td>0.12-0.5</td>
<td>4</td>
<td>0.12-0.5</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.06-4</td>
<td>0.06-0.12</td>
<td>1-2</td>
<td>0.12</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.25-2</td>
<td>1</td>
<td>1-2</td>
<td>0.5-2</td>
</tr>
</tbody>
</table>

*MIC range (mg/L)
TABLE 3. Susceptibility pattern among the singleton clones

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>mef(E)/mef(A) (n=7)</th>
<th>erm(B) + mef(E) (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC (mg/L)</td>
<td>% of overall resistance</td>
</tr>
<tr>
<td></td>
<td>Range 50 90</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>2-16 4 16</td>
<td>100</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>≤0.03-0.25 0.03 0.25</td>
<td>0</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>≤0.03-0.5 0.12 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.12-4 1 4</td>
<td>42.8</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.01-1 0.03 1</td>
<td>14.2</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.008-0.25 0.01 0.25</td>
<td>0</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.5-1 1 1</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 1. Clustering of 30 ST identified within 45 mef positive Streptococcus pneumoniae isolates by use of the minimum spanning tree. Each circle represents an ST, and the type number is indicated in the circle. The area of each circle corresponds to the number of isolates. Thick, short, solid lines connect single-locus variants; thin, longer, solid lines connect double-locus variants; black dotted lines connect STs which differ in three loci; and gray dotted lines connect STs that differ in more than three loci. Clonal complexes (CC) and international resistant clones (PMEN) are indicated.