ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2008, p. 1964–1969 Vol. 52, No. 6
0066-4804/08/$08.00+0 doi:10.1128/AAC.01487-07

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

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Received 15 November 2007/Returned for modification 4 February 2008/Accepted 15 March 2008

The population structure (serotypes, pulsed-field gel electrophoresis [PFGE] types, and multilocus sequencing types) of 45 *mef*-positive *Streptococcus pneumoniae* isolates [carrying *mef* alone ($n = 17$) or with the *erm*(B) gene $n = 28$)] were studied. They were selected from among all erythromycin-resistant isolates ($n = 244$) obtained from a collection of 712 isolates recovered from different Spanish geographic locations in the prevaccination period from 1999 to 2003. The overall rates of resistance (according to the criteria of the CLSI) among the 45 *mef*-positive isolates were as follows: penicillin G, 82.2%; cefotaxime, 22.2%; clindamycin, 62.2%; and tetracycline, 68.8% [mainly in isolates carrying *erm*(B) plus *mef*(E); $P < 0.001$]. No levofloxacin or telithromycin resistance was found. Macrolide resistance phenotypes (as determined by the disk diffusion approximation test) were 37.7% for macrolide resistance [with all but one due to *mef*(E)] and 62.2% for constitutive macrolide-lincosamide-streptogramin B resistance [cMLS$_B$; with all due to *mef*(E) plus *erm*(B)]. Serotypes 14 (22.2%), 6B (17.7%), 19A (13.3%), and 19F (11.1%) were predominant. Twenty-five different DNA patterns (PFGE types) were observed. Our *mef*-positive isolates were grouped (by eBURST analysis) into four clonal complexes ($n = 18$) and 19 singleton clones ($n = 27$). With the exception of clone Spain^{9V}-3, all clonal complexes (clonal complexes 6B, Spain^{6B}-2, and Sweden^{15A}-25) and 73.6% of singleton clones carried both the *erm*(B) and the *mef*(E) genes. The international multiresistant clones Spain^{23F}-1 and Poland^{6B}-20 were represented as singleton clones. A high proportion of *mef*-positive *S. pneumoniae* isolates presented the *erm*(B) gene, with all isolates expressing the cMLS$_B$ phenotype. A polyclonal population structure was demonstrated within our Spanish *mef*-positive *S. pneumoniae* isolates, with few clonal complexes overrepresented within this collection.

Macrolide resistance among *Streptococcus pneumoniae* clinical isolates has risen to prominence. The rate of resistance to macrolides is even higher than that to penicillin, particularly in Spain and some other European countries, such as France, Poland, Greece, and Portugal (17, 23). 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 macrolide-lincosamide-streptogramin B resistance (MLS$_B$) phenotype and is associated with conjugative transposons. The clonal dispersion of *erm*(B)-positive *S. pneumoniae* isolates and the horizontal transfer of transposable elements carrying this determinant have been associated with the increase in the rate 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 endow the macrolide resistance (M) phenotype (7). This phenotype has traditionally been more prevalent in North America than in Europe (7, 12).

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

MATERIALS AND METHODS

Bacterial isolates. Seven hundred twelve *S. pneumoniae* clinical isolates (244 isolates nonsusceptible to erythromycin) recovered during the prevaccination period (in Spain, the conjugate heptavalent vaccine was accepted for use in 2002, but it was rarely used until 2004) were studied. Isolates were prospectively collected from 14 Spanish hospitals representing 14 different geographic areas

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▽ Published ahead of print on 24 March 2008.

during the fall and winter seasons of 1999 through 2003. Among these strains, a total of 45 *mef*-positive isolates were detected, and all of them were selected for further studies. The origins of these 45 isolates are included in Table 1. A total of 26.6% (12 of 45) of these isolates were of pediatric origin.

Susceptibility testing and erythromycin resistance phenotypes. The MICs of penicillin G, cefotaxime, erythromycin, clindamycin, telithromycin, tetracycline, and levofloxacin were determined by the 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 the reference strain in each run. The breakpoints were those established by the CLSI guidelines (3). The phenotypic detection of *erm* induction was performed by using the macrolide (erythromycin)-clindamycin disk diffusion approximation test, as described previously (17).

Detection of erythromycin resistance genes. A real-time PCR approach was carried out for detection of the *erm* and *mef* genes among erythromycin-resistant isolates by using the conditions described previously (20). A subsequent scheme of multiplex PCR was followed to differentiate between the *mef*(A) and the *mef*(E) genes. Specific primers designed for this purpose were forward primer mefAF (5′-AATACAACAATTGGAAACTT-3′), forward primer mefEF (5′-AAGGAGTTGTGGTTCTGA-3′), and a reverse primer for both the *mef*(A) and the *mef*(E) genes, primer mefR (5′-AATCGTGTAAATCATTGG-3′). The expected sizes of the PCR products were 1,080 kb for *mef*(A) and 480 kb for *mef*(E). The PCR amplification mixture of 25 μl contained 15 mM Tris-HCl, 50 mM KCl (pH 8.0), 25 mM $MgCl_2$, 100 μM of each nucleotide, 0.15 pmol of the two forward primers mefAF and mefEF, 0.3 pmol of reverse primer mefR, 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), and 1 μl of genomic DNA. The PCR conditions (PTC-100 thermocycler; MJ Research Inc., Watertown, MA) 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 the amplification cycles, a final elongation step of 10 min at 72°C was included.

Population structure. Serotyping was performed with the Neufeld Quellung reaction by 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 by following the standard protocol for gram-positive bacteria, with some modifications (6). The DNA was restricted with the SmaI endonuclease (Amersham Biosciences Europe GmbH, Freiburg, Germany). Electrophoresis was performed with CHEF DR-III equipment (Bio-Rad, Birmingham, United Kingdom) for 23 h at 14°C, and the following settings were applied: 6 V/cm and 1 to 30 s. The PFGE patterns obtained were compared with those for clones established by the Pneumococcal Molecular Epidemiology Network (16).

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

Statistical analysis. Statistical associations were analyzed by the chi-square test. Differences were considered statistically significant when the two-tailed *P* value was less than 0.05.

RESULTS

Macrolide resistance genes, phenotypes, and susceptibility patterns. Within the entire *S. pneumoniae* population (n = 712), 244 isolates were nonsusceptible to erythromycin. Among those isolates, 80.7% carried the *erm*(B) gene as the sole genetic determinant 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 had the *mef*(A) gene. It is of note that 28 of 45 *mef*-positive isolates also carried the *erm*(B) gene (62.2%). In two isolates displaying erythromycin and clindamycin MICs of 0.5 mg/liter, neither the *erm*(B) gene nor the *mef* gene was detected. The constitutive MLS_B phenotype was observed in all 28 isolates carrying both the *mef* and the *erm*(B) genes, whereas the M phenotype was detected in the remaining 17 isolates that carried only one of the *mef* genes alone. No temporal or local geographic association among the *mef*-positive or the *mef*- plus *erm*(B)-positive isolates was observed (data not shown).

The overall rates of resistance to penicillin G, cefotaxime, tetracycline, and clindamycin among all 45 *mef*-positive isolates were 82.2% [35.5% for *mef*-positive isolates plus 46.7% for the *mef*- plus *erm*(B)-positive isolates], 22.2% [17.7% for the *mef*-positive isolates plus 4.4% for the *mef*- plus *erm*(B)-positive isolates], 68.8% [13.3% for the *mef*-positive isolates plus 55.5% the *mef*- plus *erm*(B)-positive isolates], and 62.2% [0% for the *mef*-positive isolates plus 62.2% for the *mef*- plus *erm*(B)-positive isolates], respectively. Neither telithromycin resistance nor levofloxacin resistance was found (MIC ranges, 0.03 to 1 mg/liter and 0.25 to 2 mg/liter, respectively).

Population structure in *mef*-positive *S. pneumoniae* isolates. The serotype distribution among the 45 *mef*-positive isolates was as follows: serotype 14, 22.2%; serotype 6B, 17.7%; serotype 19A, 13.3%; serotype 19F, 11.1%; serotype 11A, 6.6%; serotype 9V, 6.6%; serotype 15A, 6.6%; serotype 23F, 4.4%; and other serotypes, 11.1%. With a single exception, all isolates belonging to serotype 14 (n = 10) harbored the *mef* gene alone. Six different PFGE patterns were found among the *mef*(E)-positive isolates, with 58.8% of them belonging to the $Spain^{9V}$-3 clone. Among the *erm*(B)- plus *mef*(E)-positive isolates, 19 different PFGE patterns were found, with the $Sweden^{15A}$-25, $Spain^{6B}$-2, $Spain^{23F}$-1, $Poland^{6B}$-20, and $Norway^{NT}$-42 clones represented. Analysis of the MLST results by use of the eBURST program showed that our *mef*-positive isolates were grouped into 4 CCs and 19 singleton clones (Fig. 1). The serotypes, the PFGE type distribution according to analysis with the eBURST program, and the corresponding STs are shown in Table 1.

The CCs grouped 18 of the *mef*-positive isolates; 10 of them carried the *mef*(E) gene as the sole resistance determinant and belonged to the $Spain^{9V}$-3 international clone, and 8 isolates carried both the *erm*(B) and the *mef*(E) genes. The latter isolates were grouped into the $Spain^{6B}$-2 international clone (n = 3); the $Sweden^{15A}$-25 clone (n = 2); and CC-6B, which includes two different STs (ST135 and ST1638, which is a double-locus variant of ST135) (n = 3).

Twenty-seven isolates were grouped into singleton clones which included some international resistant clones, such as $Spain^{23F}$-1 [two isolates harbored both the *erm*(B) and the *mef*(E) genes], $Poland^{6B}$-20 and $Norway^{NT}$-42 [one isolate each carried both the *erm*(B) and the *mef*(E) genes], and $England^{14}$-9 [represented by the only isolate that carried the *mef*(A) gene detected in this study]. With the exception of tetracycline (MIC, 4 mg/liter), this isolate was susceptible to all antibiotics tested. ST276 (a single-locus variant of the $Denmark^{14}$-32 clone), ST549, and ST62 grouped more than one isolate each (four, three, and three isolates, respectively), while the other STs were each represented by a single isolate. The relationship between the STs and the macrolide resistance genes is shown in Table 1.

Serotype distribution according to analysis with the eBURST program showed that even though strains of some serotypes belonged to specific international clones, such as serotypes 6B, 14, 19A, and 15A, these serotypes were also found among the isolates grouped as singleton clones. The

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

Gene	MLST type		PFGE type or subtype	Serotype(s) (no. of isolates)	Yr of isolation	Origin (no. of isolates)
	ST (no. of isolates)	CC or singleton clone				
mef(E)	ST557 (7)	CC-Spain^{9V}-3	Spain^{9V}-3	14 (6), 19A (1)	1999–2003	Sputum (5), ear (1), blood (1)
mef(E)	ST44 (1)	CC-Spain^{9V}-3	Spain^{9V}-3	14	1999–2003	Nasal cavity (1)
mef(E)	ST2636 (1)	CC-Spain^{9V}-3	Spain^{9V}-3	19A	1999–2003	Sputum (1)
mef(E)	ST2637 (1)	CC-Spain^{9V}-3	Spain^{9V}-3	14	1999–2003	BAL[a] fluid (1)
mef(E)	ST62 (3)	S[b]	11A-ST62	11A	1999–2003	BAL fluid(1), alveolar brush (1), conjunctiva (1)
mef(E)	ST2819 (1)	S	9V-ST2819	9V	2000–2001	Nasal cavity (1)
mef(E)	ST2708 (1)	S	31-ST2708	31	2000–2001	Conjunctiva (1)
mef(E)	ST2822 (1)	S	9V-ST2822	9V	2002–2003	Sputum (1)
mef(A)	ST9 (1)	S	England^{14}-9	14	2000–2001	Ear (1)
erm(B) + *mef*(E)	ST 90 (1)	CC-Spain^{6B}-2	Spain^{6B}-2	6B	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 1542 (1)	CC-Spain^{6B}-2	Spain^{6B}-2	6B	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 1624 (1)	CC-Spain^{6B}-2	Spain^{6B}-2	6B	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 135 (2)	CC-6B	Clone^{6B}-ST135	6B	1999–2000	Sputum (2)
erm(B) + *mef*(E)	ST 2638 (1)	CC-6B	Clone^{6B}-ST2638	6B	1999–2000	Nasal cavity (1)
erm(B) + *mef*(E)	ST 63 (1)	CC-Sweden^{15A}-25	Sweden^{15A}-25	15A	2000–2001	Blood (1)
erm(B) + *mef*(E)	ST 1149 (1)	CC-Sweden^{15A}-25	Sweden^{15A}-25	15A	2000–2001	Conjunctiva
erm(B) + *mef*(E)	ST 276 (4)	S	Clone^{19A}-ST276	19A	2000–2001	Conjunctiva (1)
erm(B) + *mef*(E)	ST 549 (3)	S	Unrelated	19F (2), 23F (1)	1999–2001	Ear (2), catheter (1)
erm(B) + *mef*(E)	ST 81 (2)	S	Spain^{23F}-1	23F, 19F	1999–2000	Bronchial aspirate (1), sputum (1)
erm(B) + *mef*(E)	ST 17 (1)	S	14-CC17	14	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 73 (1)	S	15A-CC73	15A	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 87 (1)	S	19F-CC87	19F	2000–2001	Nasal cavity (1)
erm(B) + *mef*(E)	ST 260 (1)	S	3-ST260	3	1999–2000	Pleural fluid (1)
erm(B) + *mef*(E)	ST 271 (1)	S	19F-ST271	19F	2002–2003	Sputum (1)
erm(B) + *mef*(E)	ST 315 (1)	S	Poland^{6B}-20	6B	2000–2001	Sputum (1)
erm(B) + *mef*(E)	ST 344 (1)	S	Norway^{NT}-42	9V	2000–2001	Sputum (1)
erm(B) + *mef*(E)	ST 1486 (1)	S	6B-ST1486	6B	1999–2000	Sputum (1)
erm(B) + *mef*(E)	ST 1577 (1)	S	15C-ST1577	15C	2002–2003	Sputum (1)
erm(B) + *mef*(E)	ST 2639 (1)	S	10A-ST2639	10A	2002–2003	Bronchial aspirate (1)
erm(B) + *mef*(E)	ST 2820 (1)	S	12F-ST2820	12F	2002–2003	Sputum (1)

[a] BAL, bronchoalveolar lavage.
[b] S, singleton clone.

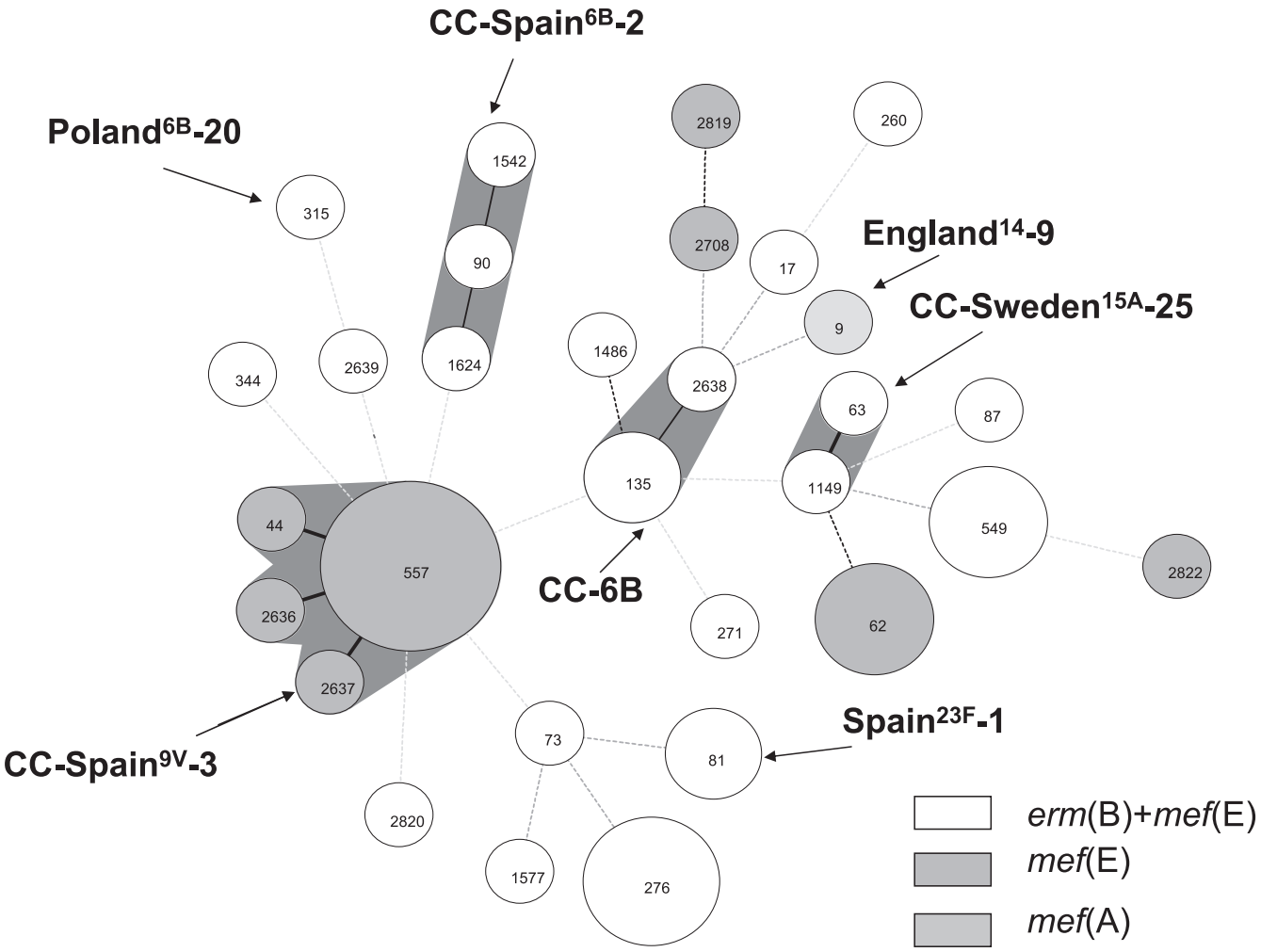

FIG. 1. Clustering of 30 STs identified among 45 *mef*-positive *Streptococcus pneumoniae* isolates by use of the minimum-spanning tree. Each circle represents an ST, and the type number is indicated in the circle. The area of each circle corresponds to the number of isolates. Thick, short, solid lines connect single-locus variants; thin, longer, solid lines connect double-locus variants; black dotted lines connect STs which differ at three loci; and gray dotted lines connect STs that differ in more than three loci. CCs and international resistant clones (Pneumococcal Molecular Epidemiology Network) are indicated.

serotype distribution according to analysis with the eBURST program is shown in Table 1.

Coresistance to penicillin (MIC range, 0.12 to 4 mg/liter) was found among the isolates grouped into CCs. The only isolate intermediate for cefotaxime (MIC, 4 mg/liter) belonged to CC-Spain^{9V}-3. This isolate also showed increased MICs for levofloxacin (2 mg/liter). This was also found among the CC-Spain^{6B}-2 and CC-6B isolates. Only eight isolates (all of them belonging to CC-Spain^{9V}-3) were susceptible to tetracycline. The susceptibility patterns among the CCs is shown in Table 2. Some differences in susceptibilities among the isolates grouped as singleton clones were observed between the *mef*-positive isolates and the isolates containing both *erm*(B) and *mef*(E).

Coresistance to penicillin G was more prevalent among the isolates carrying *erm*(B) plus *mef*(E) (89.2%) than among the *mef*(E)/*mef*(A)-positive (70.5%) isolates. The same trend, but with a high degree of statistical significance ($P < 0.001$), was observed for the rate of tetracycline resistance among the isolates positive for both *erm*(B) plus *mef*(E) (92.8%) compared with that among the *mef*(E)/*mef*(A)-positive (29.4%) isolates. The rates of cefotaxime resistance were similar between the two groups. The susceptibility patterns of the singleton clones according to the macrolide resistance genotype are shown in Table 3.

TABLE 2. Susceptibility patterns of isolates belonging to different clonal complexes

Antimicrobial agent	MIC range (mg/liter)			
	Spain^{9V}-3 *mef*(E) (n = 10)	Sweden^{15A}-25 *erm*(B) + *mef*(E) (n = 2)	Spain6B *erm*(B) + *mef*(E) (n = 3)	CC-6B *erm*(B) + *mef*(E) (n = 3)
Erythromycin	0.5–16	≥64	≥64	32–>64
Clindamycin	0.03–0.25	≥64	≥64	32–>64
Telithromycin	0.05–0.1	0.008–0.03	0.08–0.5	<0.01–0.06
Tetracycline	0.5–4	≥64	≥64	4–>64
Penicillin	0.12–4	0.12–0.5	4	0.12–0.5
Cefotaxime	0.06–4	0.06–0.12	1–2	0.12
Levofloxacin	0.25–2	1	1–2	0.5–2

TABLE 3. Susceptibility patterns of singleton clones

Antimicrobial agent	*mef*(E)/*mef*(A) (*n* = 17)				*erm*(B) + *mef*(E) (*n* = 20)			
	MIC (mg/liter)			% of overall resistance	MIC (mg/liter)			% of overall resistance
	Range	50%	90%		Range	50%	90%	
Erythromycin	2–16	4	16	100	32–>64	64	>64	100
Clindamycin	≤0.03–0.25	0.03	0.25	0	16–>64	64	>64	100
Telithromycin	≤0.03–0.5	0.12	0.5	0	<0.03–1	0.06	0.25	0
Tetracycline	0.12–4	1	4	42.8	2–>32	16	>32	95
Penicillin	0.01–1	0.03	1	14.2	≤0.015–4	1	4	85
Cefotaxime	0.008–0.25	0.01	0.25	0	≤0.015–4	0.5	2	25
Levofloxacin	0.5–1	1	1	0	0.5–1	1	1	0

DISCUSSION

Typing studies are useful for providing an understanding of the epidemiology and spread of resistant bacteria as well as establishing control protocols against epidemics (9). For *S. pneumoniae*, capsular typing is commonly used in clinical laboratories and has demonstrated that particular serotypes, such as serotypes 23F and 6B, are related to the worldwide spread of penicillin G-resistant isolates (5, 21). PFGE is useful as a means of observing recent changes, and MLST is valuable for discriminating the variations that slowly accumulate among the bacterial population (9). These techniques have been used in our study with 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, the *mef*-positive isolates among our Spanish *S. pneumoniae* isolates were demonstrated to be polyclonal, with few CCs overrepresented within the collection studied.

Resistance to macrolides in *S. pneumoniae* dramatically increased in Spain during the prevaccination period from 1999 to 2003 (17, 23). In our collection, 34.3% of the isolates were resistant to erythromycin, mainly due to the presence of the *erm*(B) gene. In recent years, an increase in the prevalence of efflux mechanisms (M phenotype) has been observed (13, 20). Among the macrolide-resistant isolates in our collection, the M phenotype was found in 6.9% of the isolates, and all of them carried a *mef* gene. However, the presence of this gene was even higher, since 11.5% (28 of 244 isolates) of the isolates with a constitutive MLS_B phenotype concomitantly presented the *erm*(B) gene. This value is higher than that previously reported from other studies in Spain, in which *mef* isolates did not have the *erm*(B) gene (2). Such an increasing association between the *mef*(E) and the *erm*(B) genes was unexpected in a country with a high incidence of *S. pneumoniae* isolates harboring the *erm*(B) gene. It is indeed difficult to understand the possible selective advantage of these isolates harboring both determinants, as *erm*(B) alone provides higher MICs than those apparently needed to resist the actions of macrolides. In other countries, the increased prevalence of isolates carrying both determinants has been related to the spread of specific clones, such the Taiwan^{19F}-14 clone, and members of a specific CC, CC-271, which includes ST271, ST236, and ST320 (8, 10). It is of note that ST271 was also found in our collection, but it was found as a singleton clone not related to the other STs (Fig. 1). In our study, the presence of isolates with both the *erm*(B) and the *mef*(E) genes was associated with three different CCs and 14 singleton clones (Table 1). These results demonstrated the nonclonal nature of the population studied and that horizontal gene transfer processes might have occurred, as may have the selection of resistant *S. pneumoniae* isolates harboring the *mef*(E) and the *erm*(B) genes.

In addition, most of the *erm*(B) plus *mef*(E) isolates were also resistant to penicillin, a fact that has also been found among isolates in geographic areas where isolates with this dual genotype are prevalent (10). On the other hand, tetracycline resistance was significant among the isolates positive for both *erm*(B) and *mef*(E). This fact could be related to the presence of conjugative transposons, like Tn*2010*, recently described in these isolates (4, 8). The participation of this trait in the maintenance of these isolates should be investigated.

Within the population with both the *erm*(B) and the *mef* determinants that we studied, we were able to identify multiresistant international clones (www.sph.emory.edu/PMEN) circulating in Spain (2, 14, 18), including clones Spain^{6B}-2 and Sweden^{15A}-25 among the CCs and clones Spain^{23F}-1, Poland ^{6B}-20 and Norway^{NT}-42 as singletons. Both the Spain^{6B}-2 and Sweden^{15A}-25 clones have been shown to be overrepresented among *S. pneumoniae* isolates that are highly resistant to penicillin or that have an MLS_B phenotype (2, 14). Among the singleton clones, the Spain^{23F}-1 clone was scarcely represented in our collection (two isolates of serotypes 19A and 23F), as were the Poland^{6B}-20 and Norway^{NT}-42 clones, compared with their representations in other studies (2, 14). Moreover, we did not find the Spain^{14}-5 clone, which also carries both genes and which has been found in some specific geographic areas of Spain over a 22-year period (15, 19). Among the isolates with the *mef* gene as the sole resistance determinant, only one isolate had the *mef*(A) subclass. This isolate belonged to the international resistant clone England^{14}-9, also described in other European countries (1). The population of *mef*(E)-positive isolates was structured into four singleton clones and a CC belonging to the Spain^{9V}-3 international resistant 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). A rate of penicillin resistance of only 14.2% was found among the *mef*(E)-positive singleton clones, and the penicillin MIC range for isolates of the CC-Spain^{9V}-3 clone was 0.12 to 4 mg/liter, in agreement with the findings of other Spanish studies (1).

In conclusion, the rate of macrolide resistance in our Spanish *S. pneumoniae* collection was high (34.3%). An increase in

the presence of the *mef* gene compared with that in other studies was detected, but this was not associated with the M phenotype. Most of the *mef*-positive isolates also harbored the *erm*(B) gene (62.2% of the *mef*- positive isolates), with all of them showing the constitutive MLS_B phenotype. Among the isolates showing the M phenotype only, one carried the *mef*(A) subclass and belonged to the England^{14}-9 clone, as previously described in Europe (1). Population structure analysis showed that the *mef*-positive isolates are grouped in different clones and CCs and not in only a few clones, as previously described in other geographic areas (1, 14).

ACKNOWLEDGMENTS

E. Gómez G. de la Pedrosa is a recipient of a post-MIR contract from the Instituto de Salud Carlos III, Ministry of Health, Spain (ref. CM07/00147). Part of this study was funded by an unrestricted grant from Sanofi-Aventis and by CIBER-ESP (the Network Center for Biomedical Research in Epidemiology and Public Health) and the Microbial Sciences Foundation.

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