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Increase of the M Phenotype among Erythromycin-Resistant *Streptococcus pneumoniae* Isolates from Spain Related to the Serotype 14 Variant of the Spain^{9V}-3 Clone

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Between 1998 and 2003 the rate of erythromycin resistance among pneumococci in Spain was 34.4%. Although the MLS_B phenotype was prevalent (94.7%), the rate of the M phenotype increased from 3.3% to 8.9% ($P < 0.01$). Clonal dissemination of *mef*(E)-carrying strains of serotype 14 variant of the Spain^{9V}-3 clone was the major contributor to this increase.

Increased resistance to macrolide in *Streptococcus pneumoniae* has been described worldwide (8–10, 14–16, 18–21, 23–27). In Europe, Mediterranean countries have the highest rates of erythromycin-resistant pneumococci, whereas northern European countries have the lowest (14–16, 20, 23, 29). In Spain, the rate of erythromycin resistance has increased progressively, from 4.3% in the period from 1979 to 1990 (19) to 22.5% in the period from 1990 to 1996 (14) and 34.5% in 2001 and 2002 (26).

Macrolide resistance in pneumococci is mainly mediated by two mechanisms: enzymatic target site modifications mediated by ErmB methylases that confer the MLS_B phenotype and active drug efflux pumps encoded by *mef* genes that confer the M phenotype. Other less frequent mechanisms have also been described: point mutations in ribosomal proteins L4 and L22 or in 23S rRNA (17, 28). The MLS_B phenotype is prevalent in Spain and in most European countries, whereas the M phenotype predominates in North America, England, and Greece (1, 8, 15, 21, 29).

In this paper, macrolide-resistant pneumococci with the M phenotype from Spain were investigated.

Evolution of macrolide resistance in pneumococci. Among 15,084 pneumococci recorded at the Spanish Reference Laboratory (1998 to 2003), 5,194 (34.4%) were erythromycin resistant. By the disk diffusion method (31) 4,917 (94.7%) showed the MLS_B phenotype and 277 (5.3%) showed the M phenotype (134 isolates were from blood, 55 were from the lower respiratory tract, 20 were from patients with otitis, 21 were from patients with conjunctivitis, and 47 were from miscellaneous sources). Table 1 shows the evolution of rates of erythromycin resistance and the phenotypes. Although the MLS_B phenotype is dominant among erythromycin-resistant pneumococci, our study shows that the proportions of isolates with the M phenotype increased significantly from 3.3% in 1998 to 8.9% in 2003 ($P < 0.01$) (Table 1). Similar rates of the M phenotype were found among erythromycin-resistant isolates from children (age, <15 years) and adults (4.6% and 5.1%, respectively).

Resistance genes. Classically, two *mef* genes have been identified in pneumococci, *mef*(A) and *mef*(E), and these are carried by different genetic elements (6). The *mef*(A) gene, which is usually found in serotype 14 strains of the England^{14}-9 clone, is carried by a defective transposon (Tn*1207.1*), whereas the *mef*(E) gene, which has been identified in different serotypes and clones, is located in the MEGA element (2, 6–9, 21, 24, 34). Recently, a new *mef* gene, *mef*(I), has been described in two serotype 11A strains (sequence type 1774 [ST1774]) (4).

The *mef* gene was detected by PCR (32) in all 277 M-phenotype pneumococci studied. After digestion with BamHI (24), 242 (87.4%) isolates had the *mef*(E) gene [this approach could not differentiate between *mef*(E) and *mef*(I)] and 35 (12.6%) had the *mef*(A) gene.

Consistent with the findings of previous studies (1, 17), we found that the erythromycin MICs of *mef*(A)-carrying pneumococci were higher (range, 8 to 128 μg/ml; geometric mean, 29.0 μg/ml) than those of *mef*(E)-carrying strains (range, 2 to 128 μg/ml; geometric mean, 12.9 μg/ml). These findings could be due to structural differences in the protein or at the site of codifying genes (1).

TABLE 1. Evolution of resistance, phenotypes, and related serotypes of erythromycin-resistant pneumococci

Yr	Total no. of isolates	Overall rate (%)			No. of Ery^r isolates	% Isolates of the following phenotype		
		Ery^{ra}	Phenotype			M		MLS_B
			MLS_B	M^b		Total^b	Serotype14^b	
1998	1,844	35.7	34.5	1.2	658	3.3	1.2	96.7
1999	2,664	34.5	33.3	1.2	919	3.5	1.5	96.5
2000	2,676	34.8	33.6	1.2	931	3.3	1.9	96.7
2001	2,546	35.5	33.6	1.9	904	5.4	3.7	94.6
2002	2,831	36.2	33.5	2.7	1,025	7.4	4.8	92.6
2003	2,523	30.0	27.3	2.7	757	8.9	5.8	91.1
Total	15,084	34.4	32.6	1.8	5,194	5.3	3.2	94.7

[a] Ery^r, erythromycin resistant.
[b] Statistically significant ($P < 0.05$) increase in the rates from 1998 to 2003.

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TABLE 2. Evolution of pneumococcal genotypes related to the *mef*(A) or *mef*(E) gene

Yr	No. (%) of isolates of the indicated genotypes harboring:						
	mef(E)				*mef*(A)		
	Total	Spain^{9V}-3	11A-ST62	Other genotypes	Total	England^{14}-9	Other genotypes
1998	20	5 (25.0)	1 (5.0)	14 (70.0)	2	2	0
1999	25	10 (40.0)	3 (12.0)	12 (48.0)	7	6	1
2000	28	16 (57.1)	1 (3.6)	11 (39.3)	3	2	1
2001	45	29 (64.4)	0	16 (35.6)	4	4	0
2002	66	41 (62.1)	8 (12.1)	17 (25.8)	10	9	1
2003	58	34 (58.6)	4 (6.9)	20 (34.5)	9	8	1
Total	242	135 (55.8)	17 (7.0)	90 (37.2)	35	31 (88.6.6)	4 (11.4)

Only 30 (12.4%) of 242 *mef*(E)-carrying isolates were tetracycline resistant [positive for *tet*(M) and *int* genes by PCR (1, 7)] and had unrelated pulsed-field gel electrophoresis (PFGE) patterns. The association of the *mef*(E) and the *tet*(M) genes was reported previously (1, 25, 30), and the genetic element harboring both genes, Tn*2009*, was recently described (7).

Serotypes and genotypes related to *mef* genes. Serotyping and PFGE (SmaI) were performed with all *mef*-carrying isolates, as described previously (14, 22, 33). Twelve strains with the dominant PFGE patterns were selected for multilocus sequence typing (MLST) (12).

Nineteen different serotypes were found among 242 *mef*(E)-carrying isolates, with the most frequent being serotype 14 (55.8%), nontypeable (10.3%), and serotype 11A (7.9%). Ninety-two PFGE patterns were observed among the *mef*(E)-carrying strains, with the Spain^{9V}-3 clone (55.8%) and genotype 11A-ST62 (7.0%) being the major clones found (Table 2). Thirty-one (88.6%) of 35 *mef*(A)-carrying isolates were serotype 14 and belonged to the England^{14}-9 clone (Tables 2 and 3).

Five of 26 international clones described by the Pneumococcal Molecular Epidemiology Network (22) were resistant to macrolides, with the resistance encoded by *mef* genes. Two of them, England^{14}-9 and Taiwan^{19F}-14 (13, 21), are the major contributors to the worldwide dissemination of M-phenotype strains (http://www.mlst.net). In contrast, our results show that the majority (48.7%, 135/277) of the M-phenotype pneumococci isolated in Spain belonged to the Spain^{9V}-3 clone and harbored the *mef*(E) gene, whereas only 11.2% (35/277) of the strains belonged to the England^{14}-9 clone and harbored the *mef*(A) gene (Table 2). No Taiwan^{19F}-14 clone was observed among the 16 serogroup 19 isolates studied.

The Spain^{9V}-3 clone was first identified in 1987 in Spain and France and is now one of the most important invasive pneumococci worldwide (www.mlst.net) (5). Strains of this clone are usually resistant to penicillin and co-trimoxazole; however, since 1998 some strains of this clone have acquired the *mef*(E) gene (20). In the United States, only 37 (14.1%) of *mef*(E)-carrying strains were related to the Spain^{9V}-3 clone (21), and all but one were of serotype 9V. In contrast, we found that 96.3% of the *mef*(E)-carrying pneumococci of the Spain^{9V}-3 clone were serotype 14, demonstrating a phenomenon of capsular switching between serotypes 9V and 14.

The England^{14}-9 clone harboring *mef*(A) has been described as predominant among M-phenotype pneumococci isolated in England, Italy, and Greece (1, 6, 15, 24). In our study this clone ranked second. In contrast, the strains of the England^{14}-9 clone described in the United States carried the *mef*(E) gene (21).

Strains of serotype 11A with ST62 have previously been found in Spain among erythromycin-susceptible pneumococci causing meningitis (11). Our results show that isolates of ge-

TABLE 3. Characteristics of strains selected for MLST

Strain	RP[a]	Serotype	Genotype	Source	*mef* gene	Allele							ST
						aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	
67/02	PESxT	14	Spain^{9V}-3	Conjunctivitis	E	7	11	10	1	6	8	1	156
544/02	PESxT	14	Spain^{9V}-3	Blood	E	7	5	10	1	6	8	1	144[b]
673/02	PESxT	14	Spain^{9V}-3	Blood	E	7	11	10	1	6	8	1	156
1615/02	PESxT	14	Spain^{9V}-3	Sputum	E	7	11	10	1	6	8	1	156
25/99	PESxT	14	Spain^{9V}-3	Blood	E	7	11	10	1	6	8	1	156
485/00	PESxT	14	Spain^{9V}-3	Sputum	E	7	11	10	1	6	8	1	156
1529/98	ESxT	11A	11A-ST62	Pus	E	2	5	29	12	16	3	14	62
2472/02	ESxT	11A	11A-ST62	Blood	E	2	5	29	12	16	3	14	62
413/02	E	14	England^{14}-9	Blood	A	1	5	4	5	5	1	8	9
1738/02	E	14	England^{14}-9	CSF[c]	A	1	5	4	5	5	1	8	9
2184/01	E	14	England^{14}-9	CSF	A	1	5	4	5	5	1	8	9
3208/02	E	14	England^{14}-9	Blood	A	1	5	4	5	5	1	5	9

[a] RP, resistance pattern; P, penicillin nonsusceptible (MIC $\geq$ 0.12 μg/ml); E, erythromycin nonsusceptible (MIC $\geq$ 1 μg/ml); SxT, co-trimoxazole nonsusceptible (MIC $\geq$ 1/19 μg/ml).

[b] Single-locus variant of ST156.

[c] CSF, cerebrospinal fluid.

notype 11A-ST62, which harbor the *mef*(E) gene, rank third among the M-phenotype pneumococci isolated in Spain. Strains of serotype 11A harboring the *mef*(A) gene have been sporadically described in Italy (23), and strains of serotype 11A harboring the *mef*(E) gene have been described in Hungary (9) and Canada (34).

In common with other findings (34), our results show that a third of the *mef*(E) isolates had unrelated genotypes, thus suggesting the horizontal spread of this gene. We previously reported a high prevalence of macrolide resistance mediated by the *mef*(E) gene in viridans group streptococci (2), which may act as a reservoir of the *mef*(E) gene and contribute to the horizontal transmission of macrolide resistance in pneumococci, as is the case in other resistance genes (3).

In conclusion, although the *mef*(E) gene may spread horizontally, the clonal dissemination of the serotype 14 variant of the Spain^{9V}-3 clone harboring the *mef*(E) gene is a major contributor to the emergence of M-phenotype pneumococci in Spain.

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