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Serotypes, Clones, and Mechanisms of Resistance of Erythromycin-Resistant *Streptococcus pneumoniae* Isolates Collected in Spain▽

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The aim of this study was to analyze the distributions of antibiotic susceptibility patterns, serotypes, phenotypes, genotypes, and macrolide resistance genes among 125 nonduplicated erythromycin-resistant *Streptococcus pneumoniae* clinical isolates collected in a Spanish point prevalence study. The prevalence of resistance to macrolides in this study was 34.7%. Multiresistance (to three or more antimicrobials) was observed in 81.6% of these strains. Among 15 antimicrobials studied, cefotaxime, moxifloxacin, telithromycin, and quinupristin-dalfopristin were the most active drugs. The most frequent serotypes of erythromycin-resistant isolates were 19F (25%), 19A (17%), 6B (12%), 14 (10%), and 23F (10%). Of the 125 strains, 109 (87.2%) showed the MLS_B phenotype [103 had the *erm*(B) gene and 6 had both *erm*(B) and *mef*(E) genes]. Sixteen (12.8%) strains showed the M phenotype [14 with *mef*(E) and 2 with *mef*(A)]. All isolates were tested by PCR for the presence of the *int*, *xis*, *tnpR*, and *tnpA* genes associated with conjugative transposons (Tn*916* family and Tn*917*). Positive detection of *erm*(B), *tet*(M), *int*, and *xis* genes related to the Tn*916* family was found in 77.1% of MLS_B phenotype strains. In 16 strains, only the *tndX*, *erm*(B), and *tet*(M) genes were detected, suggesting the presence of Tn*1116*, a transposon recently described for *Streptococcus pyogenes*. Five clones, namely, Sweden^{15A}-25, clone^{19F} ST87, Spain^{23F}-1, Spain^{6B}-2, and clone^{19A} ST276, accounted for half of the MLS_B strains. In conclusion, the majority of erythromycin-resistant pneumococci isolated in Spain had the MLS_B phenotype, belonged to multiresistant international clones, and carried the *erm*(B), *tet*(M), *xis*, and *int* genes, suggesting the spread of transposons of the Tn*916* family.

Streptococcus pneumoniae is an important human pathogen associated with respiratory tract infections, bacteremia, and meningitis (28, 30). Antimicrobial resistance among *S. pneumoniae* has spread worldwide, and an increase in erythromycin resistance has also been described (21). Data from a global international surveillance project (PROTEKT, 1993–2003) showed an increase in the global rate of macrolide resistance, from 31.0% in 1999 to 36.3% in 2003 (35), but important differences in these rates were found among countries. In Europe in 2003, the highest rates of macrolide resistance were observed in Greece (55.1%), France (54.2%), Italy (41.8%), Belgium (31.3%), and Spain (30.8%), whereas the lowest rates were found in Czech Republic (3.9%), The Netherlands (4.5%), and Sweden (6.8%). In North America, the prevalence of resistance to macrolides was higher in the United States (35.4%) than in Mexico (27.5%) or Canada (14.7%). Most alarming are the resistance rates found in Far East countries, from 68.3% in China to 79.3% in Japan and 91.2% in Taiwan (35). Coresistance to macrolide and beta-lactam antibiotics is a frequent finding among pneumococci of serotypes 6A, 6B, 14, 15A, 19F, 19A, 23F, and 23A, and these multidrug-resistant strains belong to international clones described by the Pneumococcal Molecular Epidemiology Network (26, 33; http://www.sph.emory.edu/PMEN).

Macrolide resistance in *S. pneumoniae* is mediated mainly by two mechanisms, namely, target site modification and an efflux pump (22). Target site modification by methylases encoded mainly by the *erm*(B) gene is related to the MLS_B phenotype (resistance to macrolide-lincosamide-streptogramin B). The second mechanism is an efflux pump encoded by *mef* genes and related to the M phenotype (resistance to 14- and 15-membered ring macrolides). Other, less commonly described mechanisms are mutations in the 23S rRNA gene and/or alterations in riboproteins L4 and L22 (22). In Spain (31) and the majority of European countries, the MLS_B phenotype is dominant, whereas the M phenotype is more frequent in England (1), Germany (33), the United States (13), and Canada (32).

In pneumococci and related streptococci, the frequent association of erythromycin and tetracycline resistance is related to transposons such as Tn*1545*, Tn*3872*, and Tn*6002*, resulting from the insertion of the *erm*(B) gene into the Tn*916* family of conjugative transposons that harbor the *tet*(M) gene (5, 9, 34). Tn*916* is a well-known transposon that carries the *tet*(M) gene and has the *int* gene (integrase) and the *xis* gene (excisionase), which encode transposition functions.

The efflux pump mechanism in pneumococci is codified by three subclasses of *mef*(A) genes, including *mef*(E), *mef*(A), and the recently described subclass *mef*(I) (8). The *mef*(E)

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gene is the most frequently found and is carried by the macrolide efflux genetic assembly (mega) element, whereas *mef*(A) is carried by a defective transposon (Tn*1207.1*) and is mainly associated with the England^{14}-9 clone (1, 11). Recently, two new composite elements of the Tn*916* family, containing the *tet*(M) gene plus mega (Tn*2009*) and the *tet*(M) and *erm*(B) genes plus mega (Tn*2010*), have been described (12).

The aim of this study was to analyze the distributions of antibiotic susceptibility patterns, serotypes, phenotypes, genotypes, and macrolide resistance genes among 125 nonduplicated erythromycin-resistant *Streptococcus pneumoniae* clinical isolates collected in a Spanish point prevalence study.

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MATERIALS AND METHODS

Bacterial strains. A multicenter point prevalence study was carried out in Spain during a 1-week period (16 to 22 February 2004), with 147 Spanish hospitals enrolled. Three hundred sixty *Streptococcus pneumoniae* isolates were collected from clinical samples from 112 centers (4). One hundred twenty-five (34.7%) of them were erythromycin resistant and were analyzed in this study. Thirty-three strains (26.4%) were isolated from invasive sites (22 blood, 6 cerebrospinal fluid, 2 peritoneal fluid, 1 joint fluid, 1 pleural fluid, and 1 aqueous humor sample), and 92 (73.6%) were isolated from noninvasive sites (54 upper respiratory tract, 16 middle ear fluid, 11 conjunctiva, 9 nasopharyngeal exudate, and 2 sinus exudate samples). *S. pneumoniae* isolates were identified by bilis solubility and optochin susceptibility. Serotyping was carried out by the Quellung reaction and/or dot blot assay, with the use of antisera provided by the Statens Seruminstitute (Copenhagen, Denmark), as described previously (17, 18).

Antibiotic susceptibility testing. MICs were determined for all pneumococcal strains by the microdilution method with Mueller-Hinton broth containing 2 to 5% lysed horse blood according to the CLSI method (7), using commercially available panels (STRHAE1; Sensititre, West Sussex, England). MICs of telithromycin were determined by a microdilution method by using commercially available panels (EMIZAQS; Sensititre, West Sussex, England). *S. pneumoniae* ATCC 49619 was used for quality control. The following antibiotics (range of dilutions) were tested: penicillin (0.03 to 8 μg/ml), amoxicillin-clavulanic acid (0.5/0.25 to 8/4 μg/ml), cefotaxime (0.06 to 4 μg/ml), erythromycin (0.25 to 32 μg/ml), azithromycin (0.5 to 4 μg/ml), josamycin (0.5 to 2 μg/ml), clindamycin (0.25 to 0.5 μg/ml), levofloxacin (0.5 to 4 μg/ml), moxifloxacin (0.25 to 1 μg/ml), trimethoprim-sulfamethoxazole (0.5/9.5 to 2/38 μg/ml), chloramphenicol (2 to 8 μg/ml), tetracycline (2 to 4 μg/ml), vancomycin (0.25 to 8 μg/ml), quinupristin-dalfopristin (1 to 2 μg/ml), and telithromycin (0.015 to 32 μg/ml). Interpretative criteria for josamycin are not given by the CLSI (6), and thus we used the breakpoints for josamycin given by the Société Française de Microbiologie (www.sfm.asso.fr). Kanamycin resistance was detected by disk diffusion using standard disks with 1 mg of kanamycin, and interpretive criteria were according to the Société Française de Microbiologie (www.sfm.asso.fr). Telithromycin resistance was also studied by the disk diffusion method by using standard disks with 15 μg of telithromycin.

Phenotypic characterization of macrolide resistance was performed by the double-disk diffusion method, using standard disks of erythromycin (15 μg) and clindamycin (2 μg). Inducible resistance to clindamycin was detected by placing the clindamycin disk 12 mm from the edge of the erythromycin disk. After incubation, organisms that showed flattening of the clindamycin zone adjacent to the erythromycin disk ("D zone") had an inducible macrolide resistance phenotype, whereas those strains that had a conserved inhibition zone with the clindamycin disk were considered to have the M phenotype (6). The tetracycline susceptibility of tetracycline-susceptible strains harboring the *tet*(M) gene was confirmed by determining MICs, with and without tetracycline induction (0.05 μg/ml), as described previously (5).

Detection of resistance genes. Macrolide resistance genes [*erm*(B), *erm*(A) subclass *erm*(TR), and *mef*(A/E)] were detected by PCR, using previously described primers and conditions (37). The PCR products of the *mef* gene were digested with BamHI (Invitrogen) in order to discriminate between the *mef*(A) and *mef*(E) gene subclasses (27). This approach was unable to differentiate between *mef*(I) and *mef*(E) genes (8).

The tetracycline resistance gene *tet*(M) and the promoter of the *aph3′-III* gene were studied by PCR by using previously described primers and conditions (1, 19). To investigate the presence of the Tn*916* family of transposons, the *int* and *xis* genes associated with this family of transposons were studied by PCR as previously described (1, 5). The presence of *tnpA* and *tnpR* genes related to the Tn*917* transposon was detected by PCR as previously described (5). Strains with a negative PCR result for all four genes related to Tn*916* or Tn*917* were tested for the presence of the resolvase gene (*tndX*) of Tn*5397* (a defective Tn*916*-related transposon) as described previously (5).

Typing methods. All erythromycin-resistant strains were typed by pulsed-field gel electrophoresis (PFGE). Genomic DNA embedded in agarose plugs was restricted with SmaI, fragments were separated by PFGE as described previously, and PFGE patterns were compared with those of clones established by the Pneumococcal Molecular Epidemiology Network (26).

Multilocus sequence typing (MLST) was performed for selected strains of each dominant PFGE pattern as described previously (14). One representative strain of each dominant PFGE pattern was selected for MLST. In addition, a representative strain of each serotype was studied for those clones showing capsular switching. The allele numbers and sequence types (STs) were assigned using the pneumococcal MLST website (http://www.mlst.net), which is maintained at Imperial College London and funded by the Wellcome Trust.

Statistical analysis. Data were recorded and stored in a database. All statistical analysis was performed using SPSS, version 10.0 (SPSS, Chicago, IL). The χ^2 test was used for statistical analysis, with Yates's correction when appropriate.

RESULTS

Susceptibility test and serotype distribution. The rate of erythromycin resistance among pneumococci recovered in the point prevalence multicenter study was 34.7% (125/360 isolates). Among the 125 Ery^r isolates, 45 were recovered from children (31 isolates from ≤2-year-old children, 10 from 3- to 5-year-old children, and 4 from 6- to 15-year-old children) and 80 were recovered from adults (40 isolates from 16- to 64-year olds and 40 isolates from ≥65-year-old adults). No statistically significant difference was found among the erythromycin resistance rates for pneumococci isolated from children (40.9% [45/110 isolates]) and from adults (32.0% [80/250 isolates]) ($P = 0.1$). However, strains isolated from ≤2-year-old children had a higher erythromycin resistance rate (68.9% [31/45 isolates]) than did those found in adults (32.0% [80/250 isolates]) ($P < 0.01$) or 6- to 15-year-old children (19.1% [4/21 isolates]) ($P < 0.01$). Similar rates of erythromycin resistance were found among invasive and noninvasive pneumococci (33/110 isolates [30.0%] versus 92/250 isolates [36.8%]; $P = 0.1$). The rate of erythromycin resistance was significantly greater among penicillin-resistant than among penicillin-susceptible strains (62.8% [93/148 isolates] versus 15.1% [32/212 isolates]; $P < 0.001$). The erythromycin-resistant isolates were associated with significantly higher rates of resistance to penicillin (74.4% [93/125 isolates] versus 27.6% [65/235 isolates]), tetracycline (80.0% [100/125 isolates] versus 27.6% [65/235 isolates]), chloramphenicol (34.4% [43/125 isolates] versus 4.6% [11/235 isolates]), and cotrimoxazole (68.8% [86/125 isolates] versus 32.3% [76/235 isolates]) ($P < 0.001$ for all comparisons) than were erythromycin-susceptible isolates. Multiresistance (to ≥3 antimicrobials) was observed in 81.6% of strains. All strains were susceptible to telithromycin, with MICs ranging from 0.03 to 0.5 μg/ml. Table 1 shows the in vitro activities of 15 antimicrobials against 125 erythromycin-resistant pneumococci. Cefotaxime and moxifloxacin showed good activity against multiresistant strains, but only telithromycin, quinupristin-dalfopristin, and vancomycin were active against all strains tested. Telithromycin was the most active of the antimicrobials tested against *S. pneumoniae*, irrespective of patient age or the origin

TABLE 1. Antimicrobial susceptibilities of 125 erythromycin-resistant *S. pneumoniae* strains

Antibiotic	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)	% Intermediate isolates	% Resistant isolates	CLSI breakpoint (μg/ml)[a]		
						Sensitive	Intermediate	Resistant
Penicillin	1	2	≤0.016–8	47.2	27.2	≤0.06	0.12–1	≥2
Amoxicillin-clavulanic acid	≤0.5/0.25	2/1	≤0.5/0.25–8/4	4.0	6.4	≤2/1	4/2	≥8/4
Cefotaxime	0.25	0.5	≤0.06–4	2.4	0.8	≤1	2	≥4
Cotrimoxazole	2/38	>2/38	≤0.5/9.5–>2/38	0.8	68.0	≤0.5/9.5	1/19–2/38	≥4/76
Tetracycline	>4	>4	≤2–>4	2.4	77.6	≤2	4	≥8
Chloramphenicol	2	>8	≤2–>8	0	34.4	≤4		≥8
Levofloxacin	1	1	≤0.5–>4	0	4.0	≤2	4	≥8
Moxifloxacin	≤0.25	≤0.25	≤0.25–>1	1.6	2.4	≤1	2	≥4
Erythromycin	>32	>32	4–>32	0	100	≤0.25	0.5	≥1
Azithromycin	>4	>4	>4	0	100	≤0.5	1	≥2
Josamycin	>2	>2	≤0.5–>2	0	87.2	≤1		≥4
Clindamycin	>0.5	>0.5	≤0.25–>0.5	0	87.2	≤0.25	0.5	≥1
Vancomycin	≤0.25	1	≤0.25–1	0	0	≤1		
Quinupristin-dalfopristin	≤1	≤1	≤1	0	0	≤1	2	≥4
Telithromycin	0.03	0.25	0.03–0.5	0	0	≤1	2	≥4

[a] Except for josamycin breakpoints, which were obtained from Société Franąise de Microbiologie (www.sfm.asso.fr).

of the specimen. Kanamycin resistance was found in 10 (8.0%) strains. Of 125 erythromycin-resistant pneumococci, 109 (87.2%) showed an MLS_B phenotype and 16 (12.8%) showed an M phenotype. The rate of the M phenotype was higher among pneumococci isolated from children under 2 years old (7/31 isolates [22.5%]) than among pneumococci isolated from adults (8/80 isolates [10%]), although the difference was not statistically significant ($P = 0.08$).

Using the double-disk test, 16 isolates showed an M phenotype, 91 isolates showed a constitutive MLS_B phenotype, and 18 showed an inducible MLS_B phenotype (Table 2). These 18 strains showed flattening of the clindamycin zone adjacent to the erythromycin disk (D zone). The MICs of erythromycin for 18 inducible MLS_B isolates ranged from 4 to >32 μg/ml, the MICs of clindamycin ranged from 0.5 to >0.5 μg/ml, and the MICs of josamycin were all >2 μg/ml. After induction with erythromycin at a subinhibitory concentration (0.05 μg/ml), all of the inducible MLS_B strains showed an increase in clindamycin MICs, and a subpopulation of resistant colonies in the inhibition zone or no inhibition zone was observed when erythromycin and clindamycin were tested again by the double-disk diffusion method.

Table 2 shows the relationships among erythromycin resistance phenotypes, MICs of erythromycin, clindamycin, and josamycin, resistance patterns, and macrolide and tetracycline resistance genes. Although 17 resistance patterns were found,

TABLE 2. Distributions of phenotypes, genotypes, and antimicrobial resistance patterns of 125 erythromycin-resistant *S. pneumoniae* isolates

Phenotype[a] (no. of isolates)	MIC range (μg/ml)			Resistance pattern[b]	No. of isolates	No. of isolates with resistance determinant			
	Erythromycin	Clindamycin	Josamycin			*erm*(B)	*mef*(E)	*mef*(A)	*tet*(M)
Constitutive MLS_B (91)	>32	>0.5	>2	Pen^r Ery^r Cli^r Jos^r Chl^r SxT^r Tet^r	26	26	1	0	26
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^r Tet^r	23	23	1	0	23
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^s Tet^r	13	13	0	0	13
				Pen^s Ery^r Cli^r Jos^r Chl^r SxT^r Tet^r	8	8	1	0	8
				Pen^s Ery^r Cli^r Jos^r Chl^s SxT^s Tet^r	8	8	1	0	8
				Pen^s Ery^r Cli^r Jos^r Chl^s SxT^r Tet^r	5	5	0	0	5
				Pen^s Ery^r Cli^r Jos^r Chl^s SxT^s Tet^s	3	3	0	0	0
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^r Tet^s	2	2	1	0	1
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^s Tet^s	1	1	0	0	0
				Pen^r Ery^r Cli^r Jos^r Chl^r SxT^s Tet^r	1	1	0	0	0
				Pen^r Ery^r Cli^r Jos^r Chl^r SxT^r Tet^s	1	1	0	0	0
Inducible MLS_B (18)	4–>32	0.5–>0.5	>2	Pen^r Ery^r Cli^r Jos^r Chl^r SxT^r Tet^r	5	5	0	0	5
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^r Tet^r	8	8	1	0	8
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^s Tet^r	2	2	0	0	2
				Pen^r Ery^r Cli^r Jos^r Chl^s SxT^s Tet^s	1	1	0	0	0
				Pen^s Ery^r Cli^r Jos^r Chl^r SxT^r Tet^r	1	1	0	0	1
				Pen^s Ery^r Cli^r Jos^r Chl^s SxT^r Tet^s	1	1	0	0	0
M (16)	2–32	≤0.25	≤0.5	Pen^r Ery^r Cli^s Jos^s Chl^s SxT^r Tet^s	6	0	6	0	0
				Pen^s Ery^r Cli^s Jos^s Chl^s SxT^s Tet^s	6	0	4	2	0
				Pen^r Ery^r Cli^s Jos^s Chl^s SxT^s Tet^s	2	0	2	0	0
				Pen^r Ery^r Cli^s Jos^s Chl^s SxT^s Tet^r	1	0	1	0	1
				Pen^r Ery^r Cli^s Jos^s Chl^r SxT^s Tet^s	1	0	1	0	0

[a] According to the results of the double-disk diffusion method (6).
[b] According to the results of the broth microdilution method.

TABLE 3. Phenotypes and genotypes among 125 erythromycin-resistant *S. pneumoniae* isolates

Phenotype (no. of isolates)	PCR detection of gene									Presumed transposon	Serotypes (no. of isolates)	PFGE pattern (no. of isolates)
	erm(B)	*mef*(E)	*mef*(A)	*tet*(M)	*aph3′-III*	*int*	*xis*	*tnpR*	*tnpA*			
MLS_B (109)	+	−	−	+	−	−	−	−	−	Tn*1116*	19F (8), 15A (4), 19A (2)	Sweden^{15A}-25 (14)[a]
	+	−	−	+	−	−	−	−	−	Tn*1116*	19F (2),[a] 6B (1)	Unrelated (3)
	+	−	−	+	−	+	+	+	+	Tn*3872*	19F (6)	Clone^{19F}-C (6)
	+	−	−	+	−	+	+	+	+	Tn*3872*	23F (3), 19A (1)	Spain^{23F}-1 (4)
	+	−	−	+	−	+	+	+	+	Tn*3872*	6B (2)	Spain^{6B}-2 (2)
	+	−	−	+	−	+	+	+	+	Tn*3872*	19A (2)	Clone^{19A}-O (2)
	+	−	−	+	−	+	+	+	+	Tn*3872*	14 (1)	Spain^{9V}-3 (1)
	+	−	−	+	−	+	+	+	+	Tn*3872*	14 (1)	Spain^{14}-5 (1)
	+	−	−	+	−	+	+	+	+	Tn*3872*	19F (2), 6A (1), 14 (1), NT (3)	Unrelated (7)
	+	−	−	+	+	+	+	+	+	Tn*917* plus Tn*1545*	19F (1)	Clone^{19F}-C (1)
	+	−	−	+	+	+	+	+	+	Tn*917* plus Tn*1545*	19A (1), NT (1)	Unrelated (2)
	+	−	−	+	−	+	+	−	−	Tn*6002*	19F (6)	Clone^{19F}-C (6)
	+	−	−	+	−	+	+	−	−	Tn*6002*	19A (1)	Clone^{19A}-O (1)
	+	−	−	+	−	+	+	−	−	Tn*6002*	23F (5), 19F (3)	Spain^{23F}-1 (8)
	+	−	−	+	−	+	+	−	−	Tn*6002*	6B (6)	Spain^{6B}-2 (6)
	+	−	−	+	−	+	+	−	−	Tn*6002*	19A (3)	Clone^{19A}- Z (3)
	+	−	−	+	−	+	+	−	−	Tn*6002*	23A (3), 23F (1)	Clone^{23A}-Y (4)
	+	−	−	+	−	+	+	−	−	Tn*6002*	14 (1)	Spain^{9V}-3 (1)
	+	−	−	+	−	+	+	−	−	Tn*6002*	6B (2)	Poland^{6B}-20 (2)
	+	−	−	+	−	+	+	−	−	Tn*6002*	19A (4), 19F (2), 23F (1), 6B (2), 16 (2), 15A (1), NT (3)	Unrelated (15)
	+	−	−	+	+	+	+	−	−	Tn*1545*	19A (4)	Clone^{19A}- Z (4)
											19A (1), 6A (1)	Unrelated (2)
	+	+	−	+	+	+	+	−	−	Tn*1545* plus mega	19A (1)	Clone^{19A}- Z (1)
	+	+	−	+	−	+	+	−	−	Tn*6002* plus mega	23F (1)	Clone^{23A}-Y (1)
	+	+	−	+	−	+	+	−	−	Tn*6002* plus mega	24 (1), 33 (1)	Unrelated (2)
	+	+	−	+	−	+	+	+	+	Tn*3872* plus mega	19F (1), 19A (1)[b]	Unrelated (2)
	+	−	−	−[b]	−	+	+	−	−		6B (1)	Spain^{6B}-2 (1)
	+	−	−	−[b]	−	+	+	−	−		11 (1), 21 (1), NT (2)	Unrelated (4)
	+	−	−	−[b]	−	−	−	−	−		23F (1), 35B (1), 5 (1)	Unrelated (3)
M (16)	−	+	−	−[b]	−	−	−	−	−	mega	14 (6)	Spain^{9V}-3 (6)
	−	+	−	−[b]	−	−	−	−	−	mega	15B (2), 29 (1), 6A (1), 6B (1), NT (2)	Unrelated (7)
	−	−	+	−[b]	−	−	−	−	−	Tn*1207.1*	14 (2)	England^{14}-9 (2)
	−	+	−	+	−	+	+	−	−	Tn*916* plus mega or Tn*2009*	NT (1)	Unrelated (1)

[a] Positive detection of *tndX* gene.
[b] Tetracycline-susceptible strains.

2 of them (Pen^r Ery^r Cli^r Chl^r SxT^r Tet^r and Pen^r Ery^r Cli^r SxT^r Tet) accounted for 49.6% of resistant strains. The majority (45%) of strains with these two resistance patterns belonged to serotype 19F.

Eighteen serotypes were found among erythromycin-resistant strains (Table 3). Five of them (19F [25%], 19A [17%], 6B [12%], 14 [10%], and 23F [10%]) accounted for 74% of all isolates. Among MLS_B phenotype strains, 64% belonged to serotypes 19F, 19A, 6B, 23F, 15A, and 14, whereas the M phenotype strains belonged mainly to serotype 14 (8 of 16 strains).

Gene detection. No *erm*(A) subclass *erm*(TR) methylase gene was detected among the 125 isolates studied. No erythromycin-resistant pneumococci were *mef* and *erm*(B) negative. Table 3 shows the associations among genotypes, serotypes, and clones. The *erm*(B) gene was detected in all 109 MLS_B isolates; 6 of them harbored both *erm*(B) and *mef*(E) genes and were isolated from five adults and one child. These six strains belonged to five serotypes (19A [two isolates], 19F, 23F, 24, and 33) and were not clonally related.

MLS_B phenotype isolates with resistance to tetracycline. The association of tetracycline and erythromycin resistance was found in 100 (91.7%) isolates with the MLS_B phenotype (Table 3). The *tet*(M) gene was detected in all of them. Twenty-seven (27.0%) tetracycline-resistant strains had *int*, *xis*, *tnpA*, and *tnpR* genes related to Tn*3872*, and 4 of these 27 isolates also carried other genes [three harbored the *aph3′-III* gene and one had a *mef*(E) gene]. Seven isolates with *int* and *xis* genes showed kanamycin resistance and positive detection of the promoter of the *aph3′-III* gene by PCR, and one of them also carried a *mef*(E) gene. This combination of genes [*erm*(B), *tet*(M), *int*, *xis*, and *aph3′-III*] is characteristic of Tn*1545*, a

TABLE 4. Properties of erythromycin-resistant strains selected for MLST

Strain	Source	PFGE pattern	Serotype	ST	Allele						
					aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*
290	Sputum	Spain^{6B}-2	6B	90	5	6	1	2	6	3	4
47	Bronchial aspirate	Poland^{6B}-20	6B	315	20	28	1	1	15	14	14
70	Cerebrospinal fluid	Spain^{9V}-3	14	156	7	11	10	1	6	8	1
236	Blood	Spain^{14}-5	14	17	1	5	4	11	9	3	47
332	Pleural fluid	England^{14}-9	14	9	1	5	4	5	5	1	8
351	Bronchial aspirate	Sweden^{15A}-25	15A	374[a]	2	5	36	12	17	1	14
78	Nose aspirate	Sweden^{15A}-25	19A	63	2	5	36	12	17	21	14
241	Sputum	Sweden^{15A}-25	19F	2100[a]	2	5	36	12	8	21	14
232	Ear aspirate	Clone^{19A}-O	19A	202[b]	8	16	19	15	6	40	26
256	Conjunctiva	Clone^{19A}- Z	19A	276	2	19	2	17	6	22	14
247	Blood	Clone^{19F}-C	19F	87	5	5	7	7	8	5	4
293	Aqueous humor	Clone^{23A}-Y	23A	42[c]	1	8	9	9	6	4	6
205	Sinus aspirate	Spain^{23F}-1	23F	81	4	4	2	4	4	1	1
249	Ear aspirate	Spain^{23F}-1	19A	81	4	4	2	4	4	1	1

[a] ST2100 and ST374 are SLV of ST63 of the Sweden^{15A}-25 clone.
[b] ST202 is a DLV of Taiwan^{19F}-14.
[c] ST42 is a DLV of Tennessee^{23F}-4.

Tn*916*-related transposon. Forty-nine (49%) isolates had *int* and *xis* genes and could be related to Tn*6002*, and three of them also carried the *mef*(E) gene. The *xis*, *int*, *tnpA*, and *tnpR* genes were not detected by PCR in the remaining 17 isolates. The *tndX* gene, a resolvase gene of a defective variant of the Tn*916* transposon (Tn*5397*), was detected in 16 of these 17 isolates, suggesting the presence of Tn*1116*, a transposon recently described for *Streptococcus pyogenes* (5).

MLS_B phenotype isolates with susceptibility to tetracycline. Five of nine tetracycline-susceptible isolates had the *erm*(B), *int*, and *xis* genes. Three of the four remaining isolates had the *erm*(B) gene alone. The last isolate had *int*, *xis*, *tnpA*, *tnpR*, *erm*(B), *mef*(E), and *tet*(M) genes and was of serotype 19A (Table 3). After induction with subinhibitory concentrations of tetracycline (5), no variations in tetracycline MIC were found for this tetracycline-susceptible *tet*(M)-positive isolate, suggesting the presence of a silent form of the *tet*(M) gene.

M phenotype isolates. Fourteen (87.5%) of 16 M phenotype isolates harbored the *mef*(E) gene, and 2 (12.5%) harbored the *mef*(A) gene. One *mef*(E) isolate was also resistant to tetracycline and harbored the *tet*(M), *xis*, and *int* genes. No *tet*(M), *xis*, and *int* genes were detected by PCR in the remaining 15 tetracycline-susceptible isolates.

Molecular typing (PFGE and MLST). Forty-nine different PFGE patterns were found among 109 MLS_B isolates (Table 3). Five of them (Sweden^{15A}-25, clone^{19F}-C, Spain^{23F}-1, Spain^{6B}-1, and clone^{19A}-Z) accounted for 51.4% of the MLS_B isolates. Among 16 M phenotype isolates, 10 different PFGE patterns were found, but two clones (Spain^{9V}-3 and England^{14}-9) accounted for half of the isolates.

Capsular switching was observed in three international clones, namely, Spain^{9V}-3, Sweden^{15A}-25, and Spain^{23F}-1. All strains of the Spain^{9V}-3 clone showed capsular switching and were serotype 14. Only 4 of 14 strains of Sweden^{15A}-25 had serotype 15A, and the remaining 10 strains showed capsular switching (8 were serotype 19F and 2 were serotype 19A). The majority of strains (8/12 strains) of clone Spain^{23F}-1 were serotype 23F, and only four strains had capsular switching (three were serotype 19F and one was serotype 19A).

To assess the identities of major PFGE patterns with global clones, 14 representative isolates of major PFGE patterns were selected for MLST (Table 4). Three isolates of the Sweden^{15A}-25 clone were related to CC63, with two single-locus variants (SLV) (ST374 and ST2100). Clone^{19A}-Z was related to ST276 (SLV of Denmark^{14}-32). Clone^{23A}-Y was related to ST42, a double-locus variant (DLV) of Tennessee^{23F}-4. Clone^{19A}-O was related to ST202, a DLV of Taiwan^{19F}-14. Clone^{19F}-C was related to ST87.

DISCUSSION

Our results show a high prevalence of resistance to macrolides in pneumococci isolated in 2004 in Spain, in agreement with previous studies (18, 31). A progressive increase in the rate of erythromycin resistance has been observed in Spain over the last 2 decades: while in 1979–1980 the pneumococcal macrolide resistance rate was 0% (23), the rate had increased to 4.3% by 1981–1989 (23), to 22.5% in 1990–1996 (18), and to 34.5% in 2001–2002 (31). Although the prevalence of resistance to macrolides in *S. pneumoniae* varies substantially among countries, the increase of macrolide-resistant pneumococci is a worldwide problem (20, 35). In Europe, the Mediterranean countries (France, Greece, Italy, and Spain) have the highest rates of erythromycin-resistant pneumococci, while the lowest resistance rates are found among northern European countries (35). These differences probably reflect variations in macrolide consumption rates (European Surveillance of Antibiotic Consumption [http://www.ua.ac.be/esac]) and the spread of multiresistant clones.

The present study shows that erythromycin-resistant isolates were associated with significantly higher rates of resistance to penicillin, tetracycline, chloramphenicol, and cotrimoxazole than were erythromycin-susceptible isolates. However, cefotaxime with in vitro activity against 98% of multiresistant pneumococci tested in this study remains a therapeutic option for nonmeningeal invasive pneumococcal infections caused by strains with MICs of $\leq$2 μg/ml (30). The resistance rates among new fluoroquinolones remained low (moxifloxacin,

2.4%; and levofloxacin, 4%). However, it is important that three strains susceptible to levofloxacin and moxifloxacin, but with ciprofloxacin MICs of 4 to 8 μg/ml, had an S79F change in ParC (data not shown), and it is well known that the development of fluoroquinolone-resistant strains during therapy is usually associated with strains with first-step mutations that were apparently susceptible to new fluoroquinolones (10). The ketolide telithromycin was active against all isolates tested, harboring the *erm*(B) gene and/or *mef* gene, inhibiting 90% of isolates at 0.25 μg/ml, in agreement with previous reports (2, 16, 35). Vancomycin and quinupristin-dalfopristin resistance was not detected among macrolide-resistant isolates.

The present study, in addition to others (26, 33), shows that resistance to macrolides and other antimicrobials is associated with the spread of multiresistant international clones of *S. pneumoniae* (http://www.sph.emory.edu/PMEN). Since the 1980s, penicillin resistance has been common in Spain (18, 23, 31) and has been related to the following five clones: Spain^{23F}-1, Spain^{6B}-2, Spain^{9V}-3, Spain^{14}-5, and clone^{19F}-ST87 (10, 14, 15, 39). All of these clones have already been associated with erythromycin and multidrug resistance (2, 3, 10, 24).

Sweden^{15A}-25 (ST63) is a worldwide disseminated clone that usually shows capsular switching with different serotypes (14, 19A, 19F, and 23F) (www.mlst.net). In our study, the Sweden^{15A}-25 clone was the most frequently found clone among erythromycin-resistant pneumococci, with 14 isolates, and the majority of isolates showed capsular switching (serotype 19F or 19A). This clone has been described in Spain as a cause of meningitis (15) and among multidrug- and ciprofloxacin-resistant strains (10). Clone^{19F}-ST87, the second in frequency, with 13 isolates, has been described previously in Spain (10, 15), Portugal, and Italy (www.mlst.net). The third and fourth most frequent clones were Spain^{23F}-1 and Spain^{6B}-2, with 12 isolates and 9 isolates, respectively. These two major clones have been prevalent in Spain in the last 2 decades (18). However, since the introduction of a seven-valent pneumococcal conjugated vaccine (PCV7) in 2001, a significant decrease in Spain^{23F}-1 and Spain^{6B}-2 clones has been observed in Spain (unpublished data). Clone^{19A}-ST276, an SLV of the Denmark^{14}-32-ST230 clone, ranked fifth in the present study. This clone has been found in other European countries, associated with serotypes/groups 14, 19, 20, 23, and 24 (www.mlst.net).

Serotypes 19F and 19A were the most frequently found serotypes among erythromycin-resistant pneumococci in this study. It is important that the majority of these isolates belonged to international clones (Sweden^{15A}-25, Denmark^{14}-32, and Spain^{23F}-1) as a result of capsular switching. These findings are in agreement with the work of Pai et al. (29), who reported an increase in the rates of infection caused by multiresistant serotype 19A pneumococci in the United States in the post-PCV7 period (2003–2004). They suggest that this increase in the rates of infection with serotype 19A may be due, in part, to serotype switching within vaccine serotype strains.

The MLS_B phenotype is prevalent in the majority of European countries, whereas the M phenotype predominates in North America (13, 32) and some European countries (England and Germany) (1, 33). Our study shows that macrolide-resistant pneumococci in Spain are associated mainly with the MLS_B phenotype, as previously reported (31, 33). However, in recent years, a significant increase in the M phenotype has been observed in Spain, from 3.3% of strains in 1998 to 8.9% of strains in 2003, associated with isolates of the serotype 14 variant of the Spain^{9V}-3 clone harboring the *mef*(E) gene (2, 3). An increase in the rate of the M phenotype (up to 12.8%) was observed in the present study, performed in 2004. This finding is in contrast with the increase of the M phenotype related to the England^{14}-9 clone harboring the *mef*(A) gene observed in Germany (38).

A worldwide emergence of pneumococci harboring both *erm*(B) and *mef*(E) genes has been described (16), with a global prevalence of 16.4% among macrolide-resistant isolates. The association of *erm*(B) and *mef*(E) genes has been related to the clonal spread of the Taiwan^{19F}-14 clone and, less frequently, to dissemination of Taiwan^{23F}-15 and Spain^{23F}-1 clones. In the present study, we found only six (5.5%) isolates that harbored both genes, but no clonal relationship among these strains was found.

Tetracycline and macrolide resistances are usually associated because the *tet*(M) and *erm*(B) genes are located in transposons of the Tn*916* family (9, 34). In agreement with this, we found that more than 90% of MLS_B strains were resistant to tetracycline and harbored both *erm*(B) and *tet*(M) genes. Our data suggest that the majority of erythromycin- and tetracycline-resistant strains could carry Tn*6002*, a Tn*916*-related transposon that harbors the *erm*(B) gene. This transposon has been detected in *Streptococcus cristatus* and *S. pyogenes* (5). The second most frequent combination of genes (*int*, *xis*, *tnpA*, and *tnpR*) found in this study could indicate the presence of Tn*3872*. This element is frequently found in pneumococci and other clinically significant streptococci (5, 19, 36) and is the result of the integration of Tn*917* into Tn*916* (25). Kanamycin resistance is related to Tn*1545*, a Tn*916* family element that acquired *erm*(B) and *aph3′III* genes. In agreement with the observations of other authors (19, 25, 36), a low prevalence of kanamycin resistance (8.0%) was detected among erythromycin- and tetracycline-resistant pneumococci.

No detection of *int*, *xis*, *tnpA*, and *tnpR* genes was observed in our study among isolates of the Sweden^{15A}-25 clone. However, all strains of this clone harbored *erm*(B), *tet*(M), and *tndX* genes. This combination of genes could be related to Tn*1116*, which was recently described for *S. pyogenes* (5). Tn*1116* is a composite structure resulting from the acquisition of the *erm*(B) gene by the Tn*916*-related defective transposon Tn*5397*, in which a resolvase gene (*tndX*) replaces the functions of the *int* and *xis* genes (5).

In conclusion, the high prevalence of macrolide resistance in Spain is due mainly to the dissemination of multiresistant pneumococcal clones and to the horizontal spread of the Tn*916* family of transposons. Further surveillance studies are needed to understand the epidemiology of macrolide-resistant pneumococci and to evaluate the impact of PCV7 in serotype and clone distributions.

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