Clonal Differences among Erythromycin-Resistant *Streptococcus pyogenes* in Spain

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The aim of this study was to determine whether the high levels of erythromycin resistance in *Streptococcus pyogenes* found in Spain are due to the introduction and spread of one or more clones. Phenotypic and genotypic techniques were used to characterize all erythromycin-resistant *S. pyogenes* (ErR) isolated in Gipuzkoa, Spain, in the last 10 years and 128 ErR isolated in Vitoria and Madrid during 1996. Of 437 ErR, 97% had the M phenotype; all 283 of the strains studied had the *mef*A determinant of resistance. After biotyping, T serotyping, *emm* typing, and genotyping, four major clones were detected. Clones B (biotype I, type T4, *emm*4, pulsed-field gel electrophoresis [PFGE] II) and D (biotype V, type T8.25, *emm*75, PFGE IV) comprised 78.8% of all ErR. The resistance of *S. pyogenes* to erythromycin was mainly due to an efflux mechanism of resistance (M phenotype); few clones were responsible for it.

The Lancefield group A streptococci (*Streptococcus pyogenes*), major causative agents of human disease (1), can produce both mild (e.g., pharyngitis) and severe (e.g., life-threatening "toxic shock-like syndrome," necrotizing fasciitis) infections. During the last few years, erythromycin-resistant *S. pyogenes* (ErR) has been reported in different parts of the world (2-4). Two distinct mechanisms of erythromycin resistance are described among group A streptococci. One consists of target-site modification by *erm* methylase (5,6) strains that express the MLS_b phenotype of resistance; the other (recently described) consists of an active drug efflux that pumps 14- and 15-membered macrolides out of the cell (7). This novel mechanism of macrolide resistance is encoded by the gene *mef*A (8), and strains show the M phenotype. In the last few years, increased resistance to erythromycin in *S. pyogenes* has been detected in Spain (9-11). Therefore, we performed an epidemiologic investigation to determine the biotypes, serotypes (T-agglutination patterns), *emm* types, and pulsed-field gel electrophoresis (PFGE) patterns of chromosomal DNA and their relationship to macrolide resistance.

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The Study

Sources of Bacterial Isolates

From 1988 to 1997, 2,561 nonduplicated isolated strains of *S. pyogenes* were collected from throat swabs and extratonsilar samples at the Nuestra Señora de Aránzazu Hospital and at primary-care centers in two districts of Gipuzkoa (approximately 300,000 residents). In 1996, two other samples were collected and included in this study; 33 ErR strains from Vitoria (Hospital Txagorritxu) and 95 from Madrid (Centro de Especialidades Argüelles). Gipuzkoa Province (San Sebastian is its capital) is located in the northeastern area of the Basque country of Spain, bordered by the Cantabric Sea and France to the north; Madrid is located in the center of Spain (415 km from San Sebastian); and Vitoria (in Alava Province) is located in the north of Spain (110 km from San Sebastian).

Identification, Susceptibility, Typing, and Clone Definition

Group A streptococci were identified by colony morphology, beta-hemolysis on blood

agar, and commercial latex-agglutination techniques (Streptex, Wellcome, Dartford, UK, or Phadebact, Boule Diagnostics AB, Huddinge, Sweden). The confirmation of *S. pyogenes* and biotyping were done with a commercially available identification system: rapid ID 32 STREP (BioMerieux, La Balme-les-Grottes, France). Biotyping was performed according to Bouvet et al. (12).

All *S. pyogenes* were tested for susceptibility to erythromycin and other antibiotics by broth microdilution. ErR strains were restudied by agar dilution and by agar diffusion (erythromycin induction of resistance) to determine macrolide and lincosamine resistance phenotypes (11).

T-protein types were determined by slide agglutination of trypsin-digested suspensions of bacteria with rabbit type-specific antiserum (SEVAC, Prague)(13).

The *emm* gene type of T-serotyped strains was determined by polymerase chain reaction (PCR)-enzyme-linked immunosorbent assay (ELISA), as described by Saunders et al. (14). Capture probes for the *emm* gene not described by Saunders et al. were selected from the DNA sequences encoding the N-terminal hypervariable region of strains of types *emm*2, *emm*9, *emm*48, and *emm*75 (GenBank accession nos. X56608, U12002, U11961, and U11993).

PFGE was done (15) with the following modifications. Cells were resuspended to an optical density $(OD)_{560nm}$ = 1.0, and 4 ml of the adjusted suspension was centrifuged. Pelleted cells were resuspended in 200 µl of Pett IV buffer (10 mM Tris, pH 7.2, 20 mM NaCl, 50 mM EDTA) and mixed with 100 µl of 2% low-melting agarose; incubation of plugs with lysozyme solution (1 mg/ml) was reduced to 3 hours. Slices of plugs were digested at 50°C for 16 hours with 30 UI of the enzyme *Sfi*I since it has proven satisfactory in differentiating DNA fragments of *S. pyogenes* (15,16). Digested inserts were electrophoresed by using a CHEF-DR III apparatus (BioRad), along with DNA size standards (BioRad) under the following conditions: 22 hours with an initial switch time of 20 seconds, rising on linear ramp to 75 seconds at 6V/cm, with an included angle of 120°C. Gels were stained with ethidium bromide and visualized under UV light with Imagestore 5000 ver.7.12 (Ultra-Violet Products Ltd, Cambridge, England). Similarities among PFGE patterns were established using the Dice coefficient and Lane-Manager 2.1 (TDI, Madrid, Spain) commercial computer software.

A clone was defined as a group of strains expressing both the same characteristic phenotype and genotype (PFGE pattern similarity ≥ 90%).

Erythromycin Resistance

We studied 2,561 strains of *S. pyogenes* isolated from 1988 to 1997 in Gipuzkoa; 309 (12.1%) were resistant to erythromycin. A report of ErR in Gipuzkoa from 1984 to 1996 (11) showed that until the end of 1990, erythromycin resistance in *S. pyogenes* was low (1.2%, 13 of 1,060); after 1990, resistance increased, reaching 34.8% (87 of 250) of all *S. pyogenes* isolated in 1995. In 1997, resistance decreased to 13.7% (57 of 417). In Madrid and Vitoria, resistance to erythromycin in 1996 was 22.4% (126 of 563) and 31.6% (43 of 136), respectively.

Among the ErR strains isolated in Gipuzkoa, two phenotypes of resistance were found: 8 (2.6%) strains showed the classic MLS_b phenotype, while the other 301 (97.4%) strains expressed the M phenotype, as shown by susceptibility testing and confirmed by the presence of the *mef*A gene (11). Of the 128 ErR isolated in Madrid and Vitoria, 5 (3.9%) strains (all from Madrid) displayed the MLS_b phenotype, while the remaining 123 (96.1%) showed the M phenotype. The presence of the *mef*A gene was searched for in 283 ErR with the M phenotype; it was detected in all.

Biotyping, T Serotyping, *emm* Typing, and PFGE of M-Phenotype ErR

Among the 424 ErR with the M phenotype, only four biotypes (of 10 possible) were identified—biotypes I, II, III, and V. In Gipuzkoa, biotype III was the only biotype found until 1990; between 1991 and 1997, biotypes I and V comprised 275 (93.8%) of the 293 resistant strains. Seven T-agglutination patterns were found in Gipuzkoa, each one correlating with an *emm* type except for TB3264 (T1 *emm*1, T2 *emm*2, T4 *emm*4, T8.25 *emm*75, T12 *emm*12, and T28 *emm*28). TB3264 biotype III was *emm*2, but TB3264 biotype I was not typeable with any of the 14 *emm* types assayed. Until 1990, type T12 *emm*12 was the only type found. Between 1991 and 1997, type T4 *emm*4 and T8.25 *emm*75 comprised 92.2% (270 of 293) of all isolates with

the M phenotype of resistance. In Vitoria and Madrid, T4 *emm*4 and T8.25 *emm*75 types were also the most frequently found.

Fifteen different PFGE patterns were found among the 424 M phenotype ErR; 92% of these strains belonged to four patterns (clones A-D) (Table, Figure 1). Among ErR with the MLS_b phenotype, eight PFGE patterns were found. Each biotype/T-serotype/*emm*-type combination corresponded with one PFGE pattern except on three occasions. Three different PFGE patterns that could be established among ErR belonged to biotype III/T12/*emm*12, two PFGE patterns belonged to I/T4/*emm*4, and another two patterns belonged to V/T8.25/*emm*75. The types of other clones, their annual distribution, and a dendrogram showing their similarities are given in the Table and Figure 2.

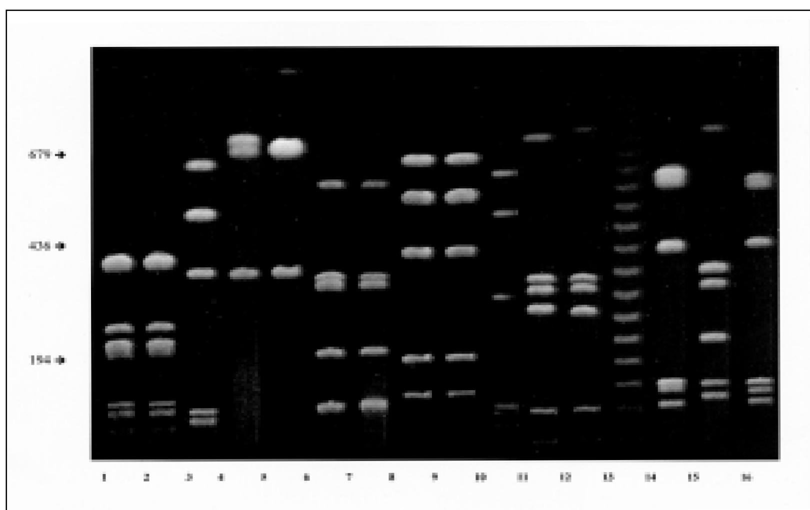

Figure 1. Pulsed-field gel electrophoresis of *Sfi*I restriction fragments of *Streptococcus pyogenes* DNAs of erythromycin-resistant S. *pyogenes*. Lanes 1-2, MLS_b phenotype strains; lane 3, clone F (biotype I, type T28, *emm*28); lanes 4-5, clone A (biotype III, type T12, *emm*12); lanes 6-7, clone B (biotype I, type T4, *emm*4); lanes 8-9, clone C (biotype I, type T4, *emm*4); lane 10, clone D (biotype V, type T8.25, *emm*75); lanes 11-12, clone E (biotype I, type T1, *emm*1); lane 13, DNA size standards (lambda ladder, 48.5 to 1,018 kb); lanes 14 and 16, clone H (biotype III, type TB3264, *emm*2); and lane 15, clone G (biotype I, type TB3264 *emm* not typeable).

Table. Annual distribution of clones of M-phenotype erythromycin-resistant *Streptococcus pyogenes*

Year	Total M-phenotype	Clones[a] A	B	C	D	E-O (biotype/T type/*emm* type)
Gipuzkoa 1988-1990	8	7				
Gipuzkoa 1991-1994	80		76			1 E (I/T1/*emm*1)
						1 F (I/T28/emm28)
Gipuzkoa 1995	83		31	18	29	1 G (I/TB3264/*emm* nt[b])
						1 H (III/TB3264/emm2)
Gipuzkoa 1996	74		19	23	25	2 H
						1 E
						1 I (I/T4/emm4)
Gipuzkoa 1997	56	1	4	3	40	2 H
						2 J (V/T8,25/emm75)
						1 K (II/T2/emm2)
						1 L (III/Tnt/emm3)
Madrid 1996	90		12	2	68	6 M (III/T12/*emm*12)
						2 N (III/T12/emm12)
Vitoria 1996	33	1	30		1	1 O ((III/Tnt/*emm*3)
Total	424[c]	9	172	46	163	23

[a]Clone A: biotype III, type T12 *emm*12, pulsed-field gel electrophoresis (PFGE) pattern I; clone B: biotype I, type T4 *emm*4, PFGE pattern II; clone C: biotype I, type T4 *emm*4, PFGE pattern III; clone D: biotype V, type T8.25 *emm*75, PFGE pattern IV.
[b]nt: nontypeable.
[c]Eleven strains were not typed.

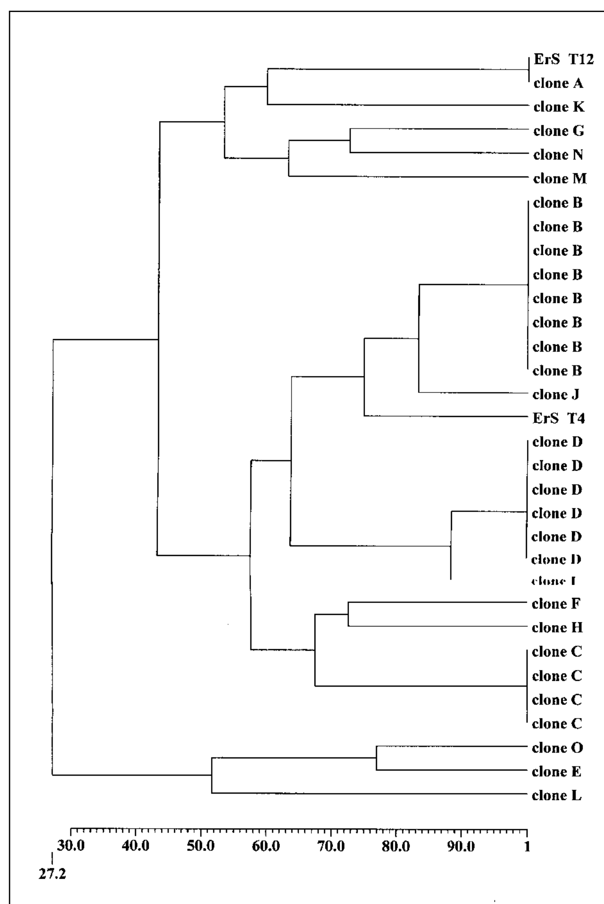

Figure 2. A dendrogram showing the genetic relationship of 15 clones of M-phenotype erythromycin-resistant *Streptococcus pyogenes* and two erythromycin-sensitive strains (ErS) established from pulsed-field gel electrophoresis patterns obtained after *Sfi*I digestion by using the Dice coefficient and UPGMA and Lane Manager 2.1 software. Clone A (III/T12/*emm*12[a]); Clone B (I/T4/*emm*4); Clone C (I/T4/*emm*4); Clone D (V/T8,25/*emm*75); Clone E (I/T1/*emm*1); Clone F (I/T28/*emm*28); Clone G (I/ TB3264/*emm* nt[b]); Clone H (III/TB3264/*emm*2); Clone I (I/T4/*emm*4); Clone J (V/T8,25/*emm*75); Clone K (II/T2/*emm*2); Clone L (III/T nt/ *emm* nt); Clone M (III/T12/*emm*12); Clone N (III/T12/*emm*12); Clone O (III/Tnt/*emm* nt); ErS T12 (III/T12/*emm*12); ErS T4 (I/T4/*emm*4).
[a]Biotype/T-serotype/*emm* type.
[b]nt = nontypeable.

Genetic Relationship of ErR and Erythromycin-Sensitive *S. pyogenes*

The genetic relationship of ErR and erythromycin-sensitive *S. pyogenes* with the same biotype and T serotype was analyzed by PFGE. In a sample of 360 erythromycin-sensitive *S. pyogenes,* biotypes I and III were the most frequent (66.7%); T1 and T28 were the most frequent T serotypes (30.6%). Only 8 (2.2%) erythromycin-sensitive strains of biotype I, serotype T4 were found. None of the 19 T8.25 erythromycin-sensitive strains found were biotype V (18 biotype II, *emm* 75 and 1 biotype I, *emm* nontypeable). Infrequent biotype and T-serotype combinations among ErR, such as I/T1 and I/T28, were frequently found among erythromycin-sensitive strains. Similarities between PFGE patterns of most of the erythromycin-sensitive and -resistant strains with the same biotype and T-serotype combination was less than 75%. Exceptions to this were several III/T12, I/T1, and I/T28 erythromycin-sensitive strains that had a close similarity (>90%) with ErR of the same biotype and T-serotype combination.

Four major clones of ErR were detected: clone A (T12, *emm*12, biotype III, PFGE I), which was present in Gipuzkoa until 1990; clone B (T4, *emm*4, biotype I, PFGE II), which was introduced in 1991; and clones C (T4, *emm*4, biotype I, PFGE III) and D (T8,25, *emm*75, biotype V, PFGE IV), which were introduced in 1995 and persisted during 1996 and 1997. In Madrid and Vitoria, 89.4% (110) of the 123 M-phenotype strains isolated in 1996 belonged to clones B and D.

Conclusions

In 1990, a new phenotype of erythromycin resistance (first designated NR and later M) was found in group A streptococci in Finland (7,17). ErR with the M phenotype had a low level of erythromycin resistance (8 mg/l to 16 mg/l) and showed cross-resistance with the 14- and 15-membered macrolides; however, they showed the same susceptibility to the 16-membered macrolides and to clindamycin as the erythromycin-susceptible strains (11). This M phenotype was prevalent among *S. pyogenes* in Europe and was the predominant phenotype of resistance among ErR isolated in Finland (18,19), Sweden (20), Austria (21), and Spain (9-11). In Italy, a high prevalence of erythromycin-resistant *S. pyogenes* was reported, but the prevalence of the M-phenotype strains among ErR varied by geographic area (22-24). Among the 437 ErR in our study in Spain, 424 (97%) showed the M phenotype; the *mef*A gene was detected and studied in 283 of these strains.

The epidemiologic surveillance of *S. pyogenes* can be done by using phenotypic or genotypic

methods or both (as we did). Biotyping does not need specialized personnel or equipment, and it is useful, in combination with serotyping methods, for a first approximation of the epidemiologic characterization of *S. pyogenes*. In this study, the most prevalent T serotypes among ErR were infrequent among erythromycin-sensitive strains and vice versa. M serotyping is a classic typing method with at least 74 types recognized, but because it is a very specialized method and reagents are not available commercially, it is restricted to a few reference centers. A rapid PCR-ELISA to determine the *emm* gene type was an accessible alternative to serology for M-antigen typing.

Although the discriminatory power of biotyping and serotyping is considered poor because different genotypes may express the same phenotypic characteristics (15,25,26), these tests were of great value in our epidemiologic surveillance. The biotype and T-serotype combination was able to discriminate between ErR and erythromycin-sensitive strains and delimited most clones among ErR.

Genomic typing methods have rarely been used in characterizing the epidemiology of noninvasive *S. pyogenes*. Among these methods, restriction endonuclease analysis of genomic DNA (REA), random amplified polymorphic DNA (RAPD), ribotyping, PFGE of chromosomal DNA, and DNA sequence analysis have been used with varying degrees of success (15,16,19,26-28). PFGE patterns confirmed the results of biotyping and serotyping and further distinguished among isolates within the same biotype and T-serotype combination. However, PFGE is a complex method—results take at least 4 days to obtain—and expensive equipment and specialized personnel are needed.

Although the polyclonal nature of the ErR strains was established in this study and previously (18), most ErR belonged to only a few clones. In Finland, 91% of the ErR isolated in 1994 were serotype T4 M4 and 88% constituted one clone by RAPD and REA (19). In our study, many clones were detected during the 10-year period. Two of the four main clones comprised 78.8% of ErR. The clonal distribution of ErR in Spain could be due to the introduction and spread of ErR from other locations or to mutations in *S. pyogenes* previously present in our environment. ErR with the M phenotype already existed in Gipuzkoa before 1990, and serotype T12 was the only one found. From 1980 to 1988, serotype T12 was predominant among ErR with the M phenotype in Sweden (20). In Gipuzkoa, the first serotype T4 ErR strain was isolated in 1991; until the end of 1994, 93.8% of ErR isolated belonged to the same clone (clone B: biotype I, type T4, *emm*4, PFGE II). Only three biotype I serotype T4 erythromycin-sensitive strains were detected before 1991. Clone B probably did not emerge in Gipuzkoa from a mutation of one of these uncommon sensitive strains. Apart from the strains described in Finland, Sweden, and Spain, strains with M phenotype were isolated in Great Britain before 1990 (4,29). Among these British and Finnish strains, type T4 M4 was frequently isolated (4,18,29). In a 1986 outbreak of 10 associated cases in Somerset, Great Britain, isolates were group A, type M4 and resistant to erythromycin (MIC 8 mg/l) but sensitive to clindamycin (29). We do not know whether the strains of clone B isolated in Spain are the same as the ErR type T4 M4 found in Great Britain and Finland before 1991, but it is probable. In Italy, serotypes T4 and T8.25 were found among M-phenotype strains (22), which suggests that a few clones have spread across Europe and caused a regional epidemic. No erythromycin-sensitive strains were detected in Gipuzkoa with the same biotype/T-type/*emm* type combination as clone D.

We believe that clones B and D, the most frequent among ErR in Spain, were of epidemic origin and that clone B probably came from northern or western Europe.

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