Molecular epidemiology, antimicrobial susceptibilities and resistance mechanisms of *Streptococcus pyogenes* isolates resistant to erythromycin and tetracycline in Spain (1994–2006)

Virginia Rubio-López, Sylvia Valdezate, David Álvarez, Pilar Villalón, María José Medina, Celia Salcedo and Juan-Antonio Sáez-Nieto

**Abstract**

**Background:** Group A *Streptococcus* (GAS) causes human diseases ranging in severity from uncomplicated pharyngitis to life-threatening necrotizing fasciitis and shows high rates of macrolide resistance in several countries. Our goal is to identify antimicrobial resistance in Spanish GAS isolates collected between 1994 and 2006 and to determine the molecular epidemiology (*emm*/T typing and PFGE) and resistance mechanisms of those resistant to erythromycin and tetracycline.

**Results:** Two hundred ninety-five out of 898 isolates (32.8%) were erythromycin resistant, with the predominance of *emm*4T4, *emm*75T25, and *emm*28T28, accounting for 67.1% of the 21 *emm*/T types. Spread of *emm*4T4, *emm*75T25 and *emm*28T28 resistant clones caused high rates of macrolide resistance. The distribution of the phenotypes was M (76.9%), cMLS\(_B\) (20.3%), iMLS\(_B\) (2.7%) with the involvement of the erythromycin resistance genes *mef*\(_A\) (89.5%), *msr*\(_D\) (81.7%), *erm*\(_B\) (37.3%) and *erm*\(_A\) (35.9%).

Sixty-one isolates were tetracycline resistant, with the main representation of the *emm*77T28 among 20 *emm*/T types. To note, the combination of *tet*\(_M\) and *tet*\(_O\) tetracycline resistance genes were similar to *tet*\(_M\) alone reaching values close to 40%. Resistance to both antibiotics was detected in 19 isolates of 7 *emm*/T types, being *emm*11T11 and the cMLS\(_B\) phenotype the most frequent ones. *erm*\(_B\) and *tet*\(_M\) were present in almost all the strains, while *erm*\(_A\), *mef*\(_A\), *msr*\(_D\) and *tet*\(_O\) appeared in less than half of them.

**Conclusions:** Spanish GAS were highly resistant to macrolides meanwhile showed minor resistance rate to tetracycline. A remarkable correlation between antimicrobial resistance and *emm*/T type was noticed. Clonal spread of *emm*4T4, *emm*75T25 and *emm*28T28 was the main responsible for macrolide resistance where as that *emm*77T28 clones were it to tetracycline resistance. A wide variety of macrolide resistance genes were responsible for three macrolide resistance phenotypes.

**Keywords:** GAS, *emm* gene, PFGE, Macrolide resistance, Tetracycline resistance
Background

Group A *Streptococcus* (GAS) causes a broad spectrum of illness in humans, ranging from pharyngitis to severe systemic diseases. A resurgence in serious GAS infections, such as rheumatic fever, and invasive diseases, such as bacteraemia, necrotising fasciitis, septic arthritis, sepsis, pneumonia and streptococcal toxic shock syndrome, has been observed since the mid 1980s. Indeed, these have become an important cause of morbidity and mortality all over the world [1].

Penicillin is the first choice treatment. Macrolides and tetracyclines are the most common alternative antibiotics used with penicillin-allergic patients or when first line therapy fails. Increases in macrolide resistance have been reported from many countries, being in Europe, very common in the Mediterranean countries [2,3].

Streptococci have two main mechanisms of macrolide resistance: target site modification and macrolide efflux systems. The first is achieved through a family of enzymes (rRNA methylases) that methylate an adenine residue (A2058) of the 23S rRNA V domain. This leads to a conformational change that reduces the binding of macrolides, lincosamide and streptogramin B to ribosomes, conferring co-resistance to these antibiotics in GAS (the MLSB phenotype). The MLSB phenotype is associated with the presence of the *erm* (erythromycin ribosome methylation) genes, with the *erm*(B) and *erm*(A) the most common [3]. In the second mechanism (the efflux system), transport proteins pump C_{14} and C_{15} macrolides out of the cell (M phenotype). The M phenotype is associated with the presence of the *mef*(A) and *msr*(D) genes, which code for the transmembrane and ATP-binding domains of this pump respectively [4].

Less information is available on the characteristics of tetracycline resistance mechanisms. In streptococci, resistance to tetracycline is conferred by ribosome protection genes such as *tet*(M) and *tet*(O) and by efflux pumps encoded by the *tet*(K) or *tet*(L) genes, although these last genes are relatively rare [4].

The prevalence of antimicrobial resistance is due to several circulating clones associated with certain *emm* types. The aim of the present study was to identify antimicrobial resistance in Spanish group A *Streptococcus* (GAS) isolates and to determine the molecular epidemiology (*emm*/T typing and PFGE) and resistance mechanisms of those resistant to erythromycin and tetracycline. This study is focused on Spanish GAS population collected from a wide spectrum of clinical backgrounds and not only from carriers as occurs for other studies. The long term studied period (13 years) and the different geographical origin may allow us to obtain an approach more real to susceptibility, phenotypes, genotypes, *emm* types and PFGE profiles distribution in Spain.

Results

Overall GAS susceptibility rates

All 898 Spanish GAS isolates showed susceptibility to penicillin and vancomycin. In addition, a 32.8% (295 isolates) rate of resistance to erythromycin was seen, along with 6.5% (59) resistance to clindamycin, 6.8% (61) resistance to tetracycline, and 0.3% (3) resistance to rifampin.

Macrolide resistance phenotypes and genotypes

Two hundred ninety five (32.8%) erythromycin resistant isolates were detected among the 898 GAS isolates gathered over the 13-year collection period. The M phenotype was clearly predominant (227 isolates, 76.9%), followed by the cMLS_{B} (60 isolates, 20.3%) and iMLS_{B} phenotypes (8 isolates, 2.7%) (Table 1). The isolates with the cMLS_{B} phenotype showed high-level resistance to erythromycin and clindamycin (MIC_{90} ≥256 mg/L), whereas those with the iMLS_{B} and M phenotypes showed lower erythromycin resistance values and susceptibility to clindamycin (Table 1). To highlight, the cMLS_{B} phenotype was more predominant among invasive that in non-invasive, 43.8 and 12.6%, respectively.

In the present work, the *mef*(A) (89.5%) and *msr*(D) (81.7%) genes were the most prevalent macrolide resistance determinants. *erm*(B) and *erm*(A) were observed in just 37.3% and 35.9% of isolates respectively (Table 1).

| Table 1 Distribution of phenotypes and genotypes among macrolide-resistant *S. pyogenes* isolates |
|---------------------------------------------------------------|
| **Phenotype** | **No. isolates (%)** | **Invasive/non-invasive** | **Antimicrobial agent (mg/L)** | **Macrolide resistance genotype** |
|----------------|-----------------------|---------------------------|-------------------------------|---------------------------------|
|                |                       |                           | **Range** | **MIC50** | **MIC90** | **erm(B)** | **erm(A)** | **mef(A)** | **msr(D)** | **None gene** |
| **M**          | 227 (76.9)            | Erythromycin              | 1-2 256 | 32        | 128       | 50        | 87        | 224        | 221        | 1             |
| 38 / 189       | Clindamycin           | 0.06/0.5                  | 0.25    | 0.5       |            |           |           |            |            |               |
| **cMLS_{B}**   | 60 (20.3)             | Erythromycin              | 8-2 256 | ≥256      | ≥256      | 57        | 11        | 36         | 17         | 2             |
| 32 / 28        | Clindamycin           | 1-2 256                   | ≥256    | ≥256      |            |           |           |            |            |               |
| **iMLS_{B}**   | 8 (2.7)               | Erythromycin              | 2-2 256 | 16        | 32        | 3         | 8         | 4          | 3          | 0             |
| 3 / 5          | Clindamycin           | 0.06/0.5                  | 0.25    | 0.5       |            |           |           |            |            |               |
| **Total**      | 295 (100)             | Erythromycin              | 1-2 256 | 64        | 256       | 110       | 106       | 264        | 241        | 3             |
| 73 / 222       | Clindamycin           | 0.06/0.5                  | 0.25    | 256       |            |           |           |            |            |               |
Fourteen macrolide resistance genotypes were identified among the 295 erythromycin-resistant isolates (Table 2), with \(\text{msr}(D)/\text{mef}(A)\) (38%) and \(\text{msr}(D)/\text{mef}(A)/\text{erm}(A)\) (19.7%) the two most common combination. Both genotypes were associated with the M phenotype.

**Tetracycline resistance phenotypes and genotypes**

Tetracycline-resistant phenotype was observed in 61 isolates (6.8%), showed MICs ranging from 8 to 64 mg/L (MIC$_{50}$ 16 mg/L, MIC$_{90}$ 32 mg/L) with a genotype distribution of \(\text{tet}(M)/\text{tet}(O)\) (42.6%), \(\text{tet}(M)\) (39.3%) and \(\text{tet}(O)\) (18.0%).

**Erythromycin and tetracycline co-resistance**

Co-resistance was detected in 19 isolates (2.1%). The erythromycin MIC was >256 mg/L for 18 isolates and just 32 mg/L for one isolate. The clindamycin MICs were also high at >256 mg/L for 14 of the 19 isolates.

Table 2 Macrolide resistance genotypes of 295 isolates of erythromycin-resistant \(S.\) pyogenes, indicating the phenotypes and \(emm/T\) types detected

| Macrolide resistance genotype | No. of isolates (%) | Phenotype $^a$ | \(emm/T\) types $^a$ |
|-----------------------------|---------------------|----------------|---------------------|
| \(\text{erm}(B)\)           | 14 (4.7)            | cMLS$_B$       |                     |
|                            |                     |                |                     |
| \(\text{erm}(B)/\text{erm}(A)\) | 1 (0.3)             | 1              | \(\text{erm}(B)/\text{erm}(A)\) (1), \(\text{erm}(B)/\text{erm}(A)\) (1) |
| \(\text{erm}(B)/\text{msr}(D)\) | 5 (1.7)             | 5              | \(\text{erm}(B)/\text{msr}(D)\) (5), \(\text{erm}(B)/\text{msr}(D)\) (5) |
| \(\text{erm}(B)/\text{mef}(A)\) | 21 (7.1)            | 20             | \(\text{erm}(B)/\text{mef}(A)\) (21), \(\text{erm}(B)/\text{mef}(A)\) (21) |
| \(\text{erm}(B)/\text{msr}(D)/\text{mef}(A)\)/ \(\text{erm}(A)\) | 33 (11.2)           | 8              | \(\text{erm}(B)/\text{msr}(D)/\text{mef}(A)\)/ \(\text{erm}(A)\) (33), \(\text{erm}(B)/\text{msr}(D)/\text{mef}(A)\)/ \(\text{erm}(A)\) (33) |
| \(\text{erm}(A)/\text{mef}(A)\) | 6 (2.0)             | 1              | \(\text{erm}(A)/\text{mef}(A)\) (6), \(\text{erm}(A)/\text{mef}(A)\) (6) |
| \(\text{erm}(A)\)           | 2 (0.7)             | -              | \(\text{erm}(A)\) (2), \(\text{erm}(A)\) (2) |
| \(\text{erm}(A)/\text{msr}(D)\) | 3 (1.0)             | -              | \(\text{erm}(A)/\text{msr}(D)\) (3), \(\text{erm}(A)/\text{msr}(D)\) (3) |
| \(\text{msr}(D)\)         | 1 (0.3)             | -              | \(\text{msr}(D)\) (1), \(\text{msr}(D)\) (1) |
| \(\text{msr}(D)/\text{mef}(A)\) | 112 (38.0)          | -              | \(\text{msr}(D)/\text{mef}(A)\) (112), \(\text{msr}(D)/\text{mef}(A)\) (112) |
| \(\text{msr}(D)/\text{mef}(A)/\text{erm}(A)\) | 58 (19.7)           | -              | \(\text{msr}(D)/\text{mef}(A)/\text{erm}(A)\) (58), \(\text{msr}(D)/\text{mef}(A)/\text{erm}(A)\) (58) |
| None gene                  | 3 (1.0)             | 2              | \(\text{None gene}\) (3), \(\text{None gene}\) (3) |

*No. of GAS isolates. $^a$ All isolates were also resistant to tetracycline, showing co-resistance to both erythromycin and tetracycline. $^b$ One isolate showed co-resistance to both erythromycin and tetracycline.
All isolates except one (iMLSB) had the cMLSB macrolide resistance phenotype. The resistance genes detected were *erm*(B) (94.7%), *erm*(A) (42.1%), *mef*(A) (47.4%), *msr*(D) (36.8%), *tet*(M) (100.0%) and *tet*(O) (36.8%), with *tet*(M) the only tetracycline resistance determinant in 13 isolates, while in 6 it was simultaneously detected with *tet*(O) (Table 3).

**T- serotypes and emm types (emm/T types) distribution**

Twenty one *emm/T* types were identified in the erythromycin-resistant population (295) (Table 3), the 6 most common being *emm*4T4 (39.3%), *emm*75T25 (14.6%), *emm*28T28 (13.2%), *emm*6T6 (9.8%), *emm*12T12 (6.8%) and *emm*11T11 (4.1%) which represented 87.8% of the erythromycin-resistant isolates. High macrolide resistance rates were associated with the above *emm/T* types: *emm*75T25 (93.5%), *emm*4T4 (84.7%), *emm*11T11 (50%), *emm*28T28 (50%), *emm*6T6 (43.3%) and *emm*12T12 (29.4%).

In the present tetracycline-resistant population (61), 20 different *emm/T* types were identified (Table 3). *emm*77T28 (37.3%) was the main *emm/T* type associated with tetracycline resistance; all *emm*77T28 isolates detected over the 13 years of the study were resistant to this antibiotic.

In the erythromycin- and tetracycline-resistant population population (19), 7 *emm/T* types were observed, the majority being *emm*11T11 (57.8%) (Table 3); indeed, 45.8% of all *emm*11T11 recovered from the initial GAS population (898) were co-resistant.

The correlation between the different *emm/T* types and macrolide resistance genotypes is shown in Table 2. The *mef*(A)/*msr*(D) gene complex was the most common in almost all *emm/T* types, either alone or in combination with other genes. The *mef*(A)/*msr*(D) genotype was the most common in the *emm*11T1 (6/10), *emm*4T4 (62/116), *emm*6T6 (26/29) and *emm*12T12 (10/20) types. The *msr*(D)/*mef*(A)/*erm*(A) (36/116) was the most common genotype among the *emm*4T4 (36/116) and *emm*75T25 (17/43) types.

**PFGE typing**

In the erythromycin-resistant population (295 isolates), 79 (26.8%) *Sma*I-restricted and 216 (73.2%) *Sma*I-non-restricted isolates were identified. *Sma*I-restricted isolates generated 30 pulsotypes with a similarity range of 38.8% to 94.7% (Figure 1). Their distribution by phenotype was: M (11 isolates), cMLS_B (58) and iMLS_B (6).

The 216 *Sma*I-non-restricted isolates (Table 4) were typed with SFl, generating 22 pulsotypes with a similarity range of 12.2% to 88.9% (Figure 2). The M phenotype (212 isolates) predominated over the cMLS_B (2) and iMLS_B (2) phenotypes. In addition, 11 different *emm/T* types were detected (Table 4) among 216 *Sma*I-non-restricted isolates, the most common being *emm*4T4 and *emm*75T25. All *emm*4T4 and all *emm*75T25 erythromycin-resistant isolates but one were *Sma*I non-restricted and had the M phenotype; together these accounted for 53.9% of the macrolide-resistant isolates in our study.

In the case of tetracycline-resistant isolates, all were *Sma*I-restricted, generating 30 pulsotypes with a similarity range of 42.16 to 100.0% (Figure 1). The *Sma*10A *emm*77T28 and *Sma*64 *emm*11T11 pulsotypes may be associated with tetracycline resistance since 100% of these isolates were resistant to this antibiotic. All co-resistant (erythromycin and tetracycline) isolates were *Sma*I-restricted.

**Discussion**

Several reports show that GAS resistance to macrolides and tetracyclines are high some countries such Spain and continue to increase; indeed, they have become clinically problematic.

In Europe, the most northerly countries (with the exception of Finland) have reported low levels of resistance (<4%) [5] while strong resistance has been reported from Mediterranean countries such as Italy (22.6%), France (22.4%), Greece (24.0%), Spain (21.3%) and Portugal (26.6%) [6-10]. This values contrast with those of Israel (1.8%) and Iran (0.2%) [11,12].

In our study, 32.8% of isolates showed resistance to macrolides. Efflux pumps (M phenotype) are one of the major mechanisms conferring resistance to macrolide antibiotics, and streptococci making use of this system have been commonly reported from European countries, Argentina, the USA and Canada [5,13-15]. The M phenotype has been identified as predominant in several Spanish studies, reaching a rate of 95.6% in a multicentre study undertaken in 1998 or 64.5% in an extensive national multicenter surveillance study in 2006–2007 [16,17]. In the present population, the efflux system was also the main macrolide resistance mechanism seen, being manifested by 76.9% of isolates.

cMLS_B phenotype, another common phenotype reported in Europe [18], was displaced by the M phenotype in several European countries from 1990 [10,19]. In our study, cMLS_B phenotype was the second most commonly encountered (20.3%) like SAUCE project carried out in 2006–2007 [17]. In this last report, fluctuations in the rates of resistance to macrolides are observed (1996–1997: 26.7%; 1998–1999: 20.4%; 2001–2002: 24.3; 2006–2007: 19%) meanwhile there is an increasing trend in the prevalence of MLS_B phenotype from 14% in 2001–2002 to 35.5% in 2006–2007 [17].

Among Spanish isolates of this work, iMLS_B phenotype was minority (2.7%) in contrast to Norway (75%)
Table 3 Distribution of emm/T types and resistance genes in *S. pyogenes* resistant to erythromycin and tetracycline with respect to the overall Spanish GAS population

| emm | T   | No. of isolates/Total | erm(B) | erm(A) | mef(A) | msr(D) | tet(M) | tet(O) |
|-----|-----|-----------------------|--------|--------|--------|--------|--------|--------|
|     |     | Erythromycin-resistant (n = 276) |        |        |        |        |        |        |
|     |     | 1 | 1 | 9/129 | 1 | 2 | 8 | 8 |
|     |     | 2 | 2 | 4/41 | 1 | 3 | 4 | 1 |
|     |     | 3 | 3 | 1/26 | 0 | 0 | 0 | 1 |
|     |     | 3 | 3/13 | 1/5 | 0 | 1 | 0 | 0 |
|     |     | 4 | 4 | 116/137 | 18 | 39 | 116 | 115 |
|     |     | 6 | 6 | 28/67 | 2 | 0 | 28 | 28 |
|     |     | 11 | 11 | 1/24 | 1 | 1 | 1 | 1 |
|     |     | 12 | 12 | 19/68 | 7 | 5 | 18 | 18 |
|     |     | 12 | NT | 1/1 | 0 | 1 | 1 | 1 |
|     |     | 22 | 12 | 3/13 | 0 | 3 | 0 | 2 |
|     |     | 28 | 28 | 37/78 | 33 | 5 | 28 | 8 |
|     |     | 28 | NT | 2/2 | 1 | 0 | 1 | 0 |
|     |     | 44 | 5/27/44 | 1/20 | 0 | 1 | 1 | 1 |
|     |     | 71 | NT | 1/1 | 1 | 0 | 0 | 0 |
|     |     | 75 | 25 | 43/46 | 19 | 32 | 42 | 42 |
|     |     | 78 | 11 | 1/30 | 1 | 0 | 0 | 0 |
|     |     | 81 | B3264 | 1/1 | 1 | 1 | 1 | 1 |
|     |     | 84 | 25 | 6/6 | 5 | 4 | 6 | 6 |
|     |     | 88 | 28 | 1/1 | 1 | 0 | 0 | 1 |
|     |     | Total | 276/898 | 92 | 98 | 255 | 234 |
|     |     | Tetracycline-resistant (n = 42) |        |        |        |        |        |        |
|     |     | 6 | 6 | 1/67 |        | 1 | 0 |
|     |     | 11 | 11 | 1/24 |        | 1 | 0 |
|     |     | 22 | 12 | 2/13 |        | 2 | 2 |
|     |     | 31/13 | NT | 1/1 |        | 1 | 1 |
|     |     | 33 | 3/13 | 1/1 |        | 1 | 0 |
|     |     | 36 | NT | 1/1 |        | 1 | 0 |
|     |     | 50 | NT | 1/2 |        | 1 | 1 |
|     |     | 58 | NT | 1/6 |        | 1 | 0 |
|     |     | 60 | 28 | 4/5 |        | 4 | 1 |
|     |     | 73 | 13 | 2/11 |        | 2 | 2 |
|     |     | 77 | 13 | 1/7 |        | 1 | 0 |
|     |     | 77 | 14/49 | 1/1 |        | 1 | 0 |
|     |     | 78 | 28 | 21/23 |        | 11 | 20 |
|     |     | 78 | 11 | 1/30 |        | 1 | 0 |
|     |     | 87 | 28 | 2/50 |        | 2 | 2 |
|     |     | NT | NT | 1/1 |        | 0 | 1 |
|     |     | Total | 42/898 | 31 | 30 |
|     |     | Erythromycin -Tetracycline-resistant (n = 19) |        |        |        |        |        |        |
|     |     | 1 | 1 | 1/129 | 1 | 0 | 1 | 1 |
|     |     | 6 | 6 | 1/67 | 1 | 0 | 0 | 0 |
|     |     | 11 | 11 | 1/24 | 11 | 3 | 3 | 5 |
|     |     | 12 | 12 | 1/68 | 1 | 1 | 1 | 1 |
Table 3 Distribution of emm/T types and resistance genes in S. pyogenes resistant to erythromycin and tetracycline with respect to the overall Spanish GAS population (Continued)

|          | emmA | emmB | emmC | emmD | emmE | emmF | emmG | emmH | emmI | emmJ |
|----------|------|------|------|------|------|------|------|------|------|------|
|          | 28   | 28   | 2    | 2    | 1    | 1    | 0    | 2    | 0    | 0    |
|          | 77   | 28   | 2/23 | 1    | 2    | 2    | 0    | 2    | 0    | 2    |
|          | 83   | NT   | 1/1  | 1    | 1    | 1    | 0    | 1    | 0    | 0    |
| Total    | 19/898 | 18 | 8    | 9    | 7    | 19   | 7    |

(1993–2002) or Bulgaria (57.7%) (1993–2002) where it was reported the most prevalent genotype [5].

A gene-phenotype correlation previously described was also noticed [3,9]. mef(A) and erm(B) were predominant in isolates with the M and cMLS B phenotype respectively, whereas all isolates with the iMLS B phenotype harboured the erm(A) gene.

The mef(A) gene responsible for the M phenotype was detected in all but three of the present Spanish isolates with that phenotype. One of these three isolates showed none of the genes studied. In the remaining two, msr(D) was observed alone or in combination with erm(A). In these last two cases, the msr(D) gene might be one of the determinants responsible for the M phenotype. msr(D) and mef(A) have been placed in the same genetic element [8,20], suggesting that the proteins they encode may act as a dual efflux system. However, it has also been suggested that the msr(D)-encoded pump can function independently of the mef-encoded protein [20].

The erm(B) gene responsible for the cMLS B phenotype was identified in all but three of the present isolates with this phenotype. None of genes tested could be amplified in two isolates, indicating that other resistance genes must be involved. The remaining isolate harboured erm (A) and mef(A). In this case, erm(A) may be responsible for the cMLS B phenotype since alterations in the regulatory region of the gene have been identified that induce constitutive expression [21].

An ample macrolide resistance genes combination was identified, specifically fourteen genotypes. Interestingly, single genotypes could show one or several phenotypes, a phenomenon reported by other authors [5,10]. One of these, emm(B)/msr(D)/mef(A) genotype showed M and MLS B phenotypes in 25 and 8 isolates respectively, while the erm(B)/erm(TR)/msr(D)/mef(A) genotype showed all three macrolide resistance phenotypes. Nowadays, this correlation between genotype and phenotype is not well understood.

In our erythromycin-resistant population (295), the 6 most common emm types: emm4T4 (39.3%), emm7T25 (14.6%), emm28T28 (13.2%), emm6T6 (9.8%), emm12T12 (6.8%) and emm11T11 (4.1%) have been previously associated with macrolide resistance in numerous reports [6,10,12,14]. emm28 and emm4 have been reported the most common in Europe (2003–2004) [18], and to be responsible for an increase in erythromycin resistance among GAS in Spain, Finland and Quebec [6]. emm12 is the main resistant emm type in Germany, Greece, Italy, Portugal, Israel [10,12,13] and the second one in the United States, being surpassed only by emm75 [14].

Most of erythromycin-resistant isolates were Sma non-restricted (73.2%) due to the presence prophage-like elements that confer the M phenotype and harbour the mef(A) and msr(D) genes. These genetic elements encode a DNA-modifying methyltransferase that acts on the SmaI recognition sequence and renders DNA refractory to cleavage by SmaI [21]. All but four of the present SmaI non-restricted isolates were susceptible to tetracycline and had an M phenotype. This suggests that these isolates carry mef(A) and msr(D) contained within a Tn1207.1 transposon inserted into a larger genetic element such as the Tn1207.3 or 58.8 kb chimeric element, flanked by the comEC gene from the Tn1207.3/Φ10394.4 family [22]. In our study, all emm4T4 and all emm75T25 erythromycin-resistant isolates but one were SmaI non-restricted and had the M phenotype; together these accounted for 53.9% of the Spanish macrolide-resistant isolates. Several resistant clones previously described in Spain were identified [9,10]. The emm4T4 Sf1 (79) clone resembles to clone B described in 1999 [10]. It was the most common in the present study, indicating it to still be circulating in Spain. This clone has a wide distribution, and it has recently been identified in Finland, Greece, Italy, England and Sweden [23]. Clone C, previously identified in Spain, the United Kingdom and the United States [23] was not detected among the present isolates, although it might be related to the present clones emm4T4 Sf4 and emm4T4 Sf5.

The major macrolide-resistant clone emm75T25 Sf12 (41) was similar (additional band between 48.5 and 97 kb) to clone D described by Perez-Trallero et al. [10]. The emm6T6 Sf17 and emm84T25 Sf22 clones might be associated with resistance since they were only observed in isolates resistant to erythromycin.

Regarding tetracycline resistance, we detected values of 6.8% between 1994 and 2006, indicating there to be no trend towards increased tetracycline in Spain. However, higher rates have been found in other countries such as Israel (23.6%), Denmark (33.7%), Portugal (38.7%) or Iran (42%) [10–12].
Figure 1 Smal-pulsotypes, emm/T and phenotypes of erythromycin- and/or tetracycline-resistant *S. pyogenes.*
In this study, a predominance of genotype with both genes tet(M) and tet(O) (42.6%) was observed. But no Spanish reports citing the predominance of both genes appears to exist, tet(M) alone is usually the most common resistance determinant followed by tet(O) [9].

In the present tetracycline-population, emm77T28 was the main emm/T type. emm77 has been previously associated with resistance to tetracycline in Israel and Europe [12]. In Italy and Norway, an emm77 clone has been reported that is characterised by its carrying tet(O) linked to erm(A)and being associated with the iMLS5 phenotype [2]. In the present study, the two co-resistant emm77T28 isolates showed genotypes different to those described by Palmieri et al. [2].

Table 4 Distribution of emm/T types, phenotypes and genotypes of erythromycin-resistant Smal-non-restricted isolates

| emm | T   | Phenotype | No. of isolates | Genotypes (no. of isolates) |
|-----|-----|-----------|----------------|-----------------------------|
|     |     |           |                | emr(B) | emr(A) | msr(D) | mef(A) |
| 1   | 1   | M         | 4              | 1      | 2      | 4      | 4      |
| 12  | 12  | M         | 16             | 4      | 2      | 16     | 16     |
| 12  | NT  | M         | 1              | 0      | 1      | 1      | 1      |
| 28  | 28  | iMLSB     | 1              | 1      | 1      | 0      | 1      |
| 4   | 4   | M         | 116            | 18     | 39     | 115    | 116    |
| 6   | 6   | M         | 27             | 1      | 0      | 27     | 27     |
| 75  | 25  | M         | 42             | 18     | 32     | 42     | 41     |
| 75  | 25  | cMLSB     | 1              | 0      | 1      | 0      | 1      |
| 81  | B3264 | iMLSB     | 1             | 1      | 1     | 1      | 1      |
| 84  | 25  | M         | 6              | 1      | 4      | 6      | 6      |
| 28  | NT  | cMLSB     | 1              | 1      | 0      | 0      | 1      |
| Total |     |           | 82             | 216    |

![PFGE-pulsotype generated with SfiI in 216 Smal-unrestricted isolates resistant only to erythromycin, indicating emm-T type.](image-url)
With regard to co-resistance, we found that all isolates (19) except one had the cMLS\textsubscript{B} macrolide resistance phenotype such as Greece (Athens) and Norway [5,15]. In contrast, in Finland, iMLS\textsubscript{B} isolates showing co-resistance have reached rates of 93% [19]. A correlation between the M phenotype and co-resistance has been also reported [23], but this was not detected in the present study.

Of the 19 co-resistant isolates, five carried \textit{tet}(M)/\textit{erm} (B) as their only resistance genes, suggesting they may carry conjugative transposons of the Tn916 family in which \textit{erm}(B) and \textit{tet}(M) are linked [24], whereas 13 harboured \textit{tet}(M)/\textit{erm}(B) associated with other resistance genes. In the remaining isolate, the \textit{erm}(B), \textit{mef}(A), \textit{tet}(M) and \textit{tet}(O) genes were all detected. \textit{mef}(A) and \textit{tet}(O) linkage has been previously reported in co-resistant isolates [22,25]. In the present work, \textit{mef}(A) appeared associated with other macrolide resistance genes and linked to \textit{tet}(M) (1 isolate) or to \textit{tet}(M)/\textit{tet}(O) (5). The main \textit{emm}/T type detected in coresistant isolates was \textit{emm}11T11 (57.8%). This \textit{emm}/T type has previously been associated with co-resistance [9,11] with an \textit{erm} (B)/\textit{tet}(M) clone prevalent among Spanish MLS\textsubscript{B} isolates [9]. Four isolates with this genotype were found in the present work, but we can not confirm whether they belong to the above clone.

Conclusion

In summary, the resistance against erythromycin, single or together to tetracycline, is due to a wide combination of resistance genes in Spanish GAS. Erythromycin resistance is mainly consequence of clonal spread of \textit{emm}4T4, \textit{emm}75T25, both associated with M phenotype and \textit{Smal} non-restricted, and \textit{emm}28T28. Whereas tetracycline resistance and co-resistance is due to clonal spread of \textit{emm}77T28 and \textit{emm}11T11, respectively, all \textit{Smal} restricted.

Methods

Bacterial isolates

Between 1994 and 2006, 898 GAS isolates were submitted for their characterisation to the Streptococcal Reference Laboratory from 75 Hospitals and Public Health Laboratories in 32 Spanish provinces. GAS identification was confirmed by colony morphology, β-haemolysis on blood agar, a latex agglutination assay (Slidex, Streptokit, BioMerieux, Marcy-L’Etoile, France), and by using the rapid ID 32 STREP kit (BioMerieux, Marcy-L’Etoile, France). The erythromycin- and tetracycline-resistant isolates were selected as the study population (see section antimicrobial susceptibility tests). This population (337 isolates) was collected from a wide spectrum of clinical backgrounds, including necrotising fasciitis (3), cellulitis and other skin infections (67), streptococcal toxic shock syndrome (13), sepsis and meningitis (17), respiratory infection (5), bone infection and rheumatic fever (4), genital infection (20), otitis (12), conjunctivitis (1), scarlet fever (70) and pharyngotonsillitis (80), as well as from asymptomatically carriers (45). For the latter status, the GAS isolates were recovered from oropharyngeal swabs. A limitation of the study was due to the voluntary nature of the submission of these strains, producing a bias in the annual number.

Antimicrobial susceptibility tests

The minimum inhibitory concentrations (MICs) of penicillin, vancomycin, erythromycin, clindamycin, tetracycline and rifampin were determined using the E-test (AB Biodisk, Solna, Sweden) following the standard method [26]. Susceptibility results were categorized according to the criteria of the Clinical and Laboratory Standards Institute [26]. The erythromycin-resistant (MIC ≥ 1 mg/L) and tetracycline-resistant (MIC ≥ 8 mg/L) isolates were then selected as the study population. \textit{Streptococcus pneumoniae} ATCC 49619 was used as control.

Detection of the macrolide resistance phenotype

Erythromycin-resistant isolates were classified on the basis of their susceptibility patterns as shown by double-disk tests involving erythromycin (15 μg) and clindamycin (2 μg) disks (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) [27]. Three phenotypes were assigned: M (erythromycin resistant and clindamycin susceptible), cMLS\textsubscript{B} (constitutive erythromycin and clindamycin resistant), and iMLS\textsubscript{B} (erythromycin resistant and clindamycin inducible). Blunting of the clindamycin inhibition zone near to the erythromycin disk indicated an iMLS\textsubscript{B} phenotype, whereas susceptibility to clindamycin with no blunting indicated the M phenotype.

Detection of erythromycin and tetracycline resistance genes

All erythromycin-resistant isolates were screened by PCR for the erythromycin resistance genes \textit{erm}(B) [28], \textit{erm}(A) [3], \textit{mef}(A) [4], and \textit{msr}(D) [29]. Tetracycline-resistant isolates were tested for the tetracycline resistance genes \textit{tet}(M) and \textit{tet}(O) [4]. PCR assays were carried out according to previously described conditions for each individual primer pairs.

T-serotype and \textit{emm} type (\textit{emm}/T types)

The T-serotype was determined by slide agglutination using type-specific antisera (Seiken-Oxoid, Cambridge, UK). \textit{emm} sequencing was performed according to the protocol of the CDC International Streptococcal Reference Laboratory (http://www.cdc.gov/ncidod/biotech/strep/protocols.html).
Pulsed field gel electrophoresis (PFGE) analysis

PFGE was performed as previously described [30] with slight modifications. Chromosomal DNA was digested with the Smal (40U) restriction enzyme (Fermentas, Vilnius, Lithuania) for 4 h at 30°C and the electrophoresis conditions were 22 h with an 0.5 to 40s switch time ramp at a 120° angle and 6 V/cm. Smal non-restricted isolates were typed by PFGE using the SfiI restriction enzyme (Fermentas, Vilnius, Lithuania) under previously described conditions [31]. The PFGE profiles were analysed using InfoQuest FP software v.4.5 (Bio-Rad Laboratories, Hercules, CA, USA), employing the UPGMA method with the Dice coefficient and a position tolerance of 1.2%. SmaI- and SfiI-profiles were number-coded. For closely related SmaI-types (1–2 bands of difference) a letter was added.

Financial competing interest

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PV, MJM, SV, JA and VR participated in the molecular data collection and analysis. DA, CS and VR conducted the microbiological methods and analysed data. SV, JA and VR interpreted data, and drafted the manuscript. SV and JA were involved in critically revising the manuscript. All authors read and approved the final manuscript.

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