Streptococcus pyogenes isolates with characterized macrolide resistance mechanisms in Spain: in vitro activities of telithromycin and cethromycin

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The in vitro activities of telithromycin and cethromycin (ABT-773) against 412 Streptococcus pyogenes isolates, consecutively collected in 17 Spanish hospitals from different geographical areas, were evaluated and compared with those of erythromycin A, penicillin G, clindamycin and quinupristin–dalfopristin. With a susceptibility testing breakpoint of \( \leq 1 \) mg/L for both compounds, 96.1% of isolates were susceptible to telithromycin and 99.8% to cethromycin. Erythromycin non-susceptible isolates (intermediate plus resistant, MIC \( \geq 0.5 \) mg/L) comprised 23% of those tested, and were analysed for the genetic basis of their resistance by PCR. Among these isolates (n = 95), 72.6% harboured mef(A), 8.4%, erm(B), and 3.2%, erm(A), as sole macrolide resistance gene, whereas the presence of mef(A) plus erm(A) (11.6%) or mef(A) plus erm(B) (4.2%) was also observed. Both ketolides displayed a significant in vitro activity against S. pyogenes regardless of the macrolide resistance mechanisms. Nevertheless, in the case of telithromycin, 11 out of 19 of the erm(B)-positive isolates (2.7% of total population) exhibited an MIC range of 4–32 mg/L. According to the present results, telithromycin and cethromycin offer a wide coverage against S. pyogenes isolates in a geographic area with a high incidence of resistance to currently used macrolides.

Keywords: S. pyogenes, ketolides, telithromycin, cethromycin, macrolide resistance

Introduction

Streptococcus pyogenes is the most common bacterial agent implicated in acute pharyngitis, and is also responsible for a variety of skin and soft tissue infections including necrotizing fasciitis and the life-threatening toxic shock syndrome.1 Macrolides constitute the alternative choice for the treatment of streptococcal pharyngitis and other respiratory tract infections when the use of penicillin is inappropriate. Nevertheless, the widespread use of macrolides has contributed to the increase in resistance among S. pyogenes populations, which is a matter of concern.2,3

Resistance to macrolide antibiotics in S. pyogenes can be ascribed to two main mechanisms: target site modification and active efflux.4 Ribosomal modification may be due to the action of methylases encoded either by erm(B) (MLS\(_B\) resistance-phenotype, inducible or constitutive) or erm(A) genes.5–4 The active drug efflux is mediated by the membrane-associated pump encoded by the mef(A) gene5,10 but a new efflux mechanism, not yet characterized, has recently been reported.11 In addition, ribosomal mutations constitute another mechanism of resistance to macrolides but this is still considered an emerging trait;12 however, even in the case of the non-transformable S. pyogenes, it could acquire relevance due to the dispersion of successful clones selected by antibiotic pressure.13 Presently described mutations comprise a C2611U change in domain V of 23S rRNA as a result of a mutation in all the six copies of \( \text{rrn} \) gene,14 and mutations in the gene coding the ribosomal protein L4 (\( \text{rplD} \)) resulting in amino acid deletions or insertions.15

Ketolides are a new class of semi-synthetic antibiotics derived from erythromycin A that retain the macrolactone ring and the \( \text{D}-\text{desosamine} \) sugar at position 5.16 The main original features reside in the presence of a 3-keto function instead of the L-cladinose moiety, responsible for the lack of induction of methylases;17 the inclusion of a C11, C12 cyclic carbamate and of an arylalkyl or an arylallyl chain linked to the molecule that improve the antibacterial activity and the pharmacodynamic properties of these compounds.18,19 These agents display the same antibacterial spectrum as macrolides but retain

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significant activity against erythromycin A-resistant streptococci, other Gram-positive strains and ‘atypical’ pathogens thus appearing to be a promising option for the treatment of infections caused by these isolates.20–23

In this study, the activities of telithromycin and cethromycin were evaluated against 412 *S. pyogenes* isolates collected during 1999–2001 in 17 Spanish hospitals. The presence of the genes implicated in macrolide resistance was investigated by PCR in erythromycin-intermediate and -resistant isolates and correlated with the resistance phenotypes obtained by MIC and disc diffusion assays.

**Materials and methods**

**Bacterial strains**

A total of 412 isolates of *S. pyogenes* was studied, prospectively collected during 1999–2001 in 17 Spanish hospitals representing different geographical areas. Of these, 210 isolates were from paediatric patients (up to 16 years old) and 202 from adults. Clinical samples were distributed as follows: nasopharynx (353), middle ear (12), blood (eight), abscess (eight), skin (six), sputum (six), other lower respiratory tract samples (six), wound (five), urine and genital specimens (four) and organic fluids (four).

**Disc diffusion assays**

Erythromycin (15 µg), clindamycin (2 µg), chloramphenicol (30 µg) and tetracycline (30 µg) discs were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK). A first screening of macrolide resistance phenotypes was carried out by the double disc diffusion test using Mueller–Hinton agar supplemented with 5% sheep blood. Erythromycin and clindamycin discs were placed on the plates 15–20 mm apart. Blunting of the clindamycin inhibition zone proximal to the erythromycin disc was interpreted as the inducible MLSₐ resistance type or the expression of *erm(A)* gene, whereas resistance to both antibiotics was considered as the constitutive MLSₐ resistance type. Resistance to erythromycin with susceptibility to clindamycin was assigned to the M phenotype (efflux). Susceptibility to chloramphenicol and tetracycline was determined by the standard agar diffusion test24 using the commercial discs cited above.

**MIC susceptibility testing**

Antibiotics were supplied as drug substance of known potency by the manufacturers indicated as follows: telithromycin (HMR 3647), erythromycin A and quinupristin–dalfopristin (Aventis Pharma, Romansville, France); clindamycin (2 µg); cethromycin (ABT-773) (Abbott Laboratories, Chicago, IL, USA); clindamycin (The Upjohn Co., Kalamazoo, MI, USA), and penicillin G (Sigma Chemical Co., St.Louis, MO, USA).

MICs were determined by the agar dilution procedure using Mueller–Hinton agar (Oxoid Ltd.) supplemented with 5% sheep blood. Plates were incubated overnight in ambient air at 35°C and standard quality control strains *S. pneumoniae* ATCC 49619 and *S. aureus* ATCC 29213 were included in each run. The MIC breakpoints recommended by the NCCLS were used for all antibiotics.25 In the case of ketolides, breakpoints proposed by the corresponding manufacturers were applied: ≤ 1 mg/L and 2 mg/L were used for susceptible and resistant category, respectively.

**Detection of erythromycin resistance genes**

DNA extraction, PCR and electrophoresis conditions were as previously described.26 Detection of *erm(B), erm(A)* and *mef(A)* genes was carried out with the following primers27 (Amersham Pharmacia Biotech, Uppsala, Sweden): *erm(B): 5′-GAA AAG GTA CTC AAC CAA ATA-3′, 5′-AGT AAC GGT ACT TAA ATT GTT TAC-3′; *erm(A): 5′-AGA AGG TTA TAA TGA AAC AGA-3′, 5′-AGT ATC ATT AAT CAC TAG TGC-3′; *mef(A): 5′-AGT ACCT ATT AAC CAG TAG TGC-3′, 5′-GGT ACG CAA ATC GTT TTC-3′). Genomic DNAs from *S. pneumoniae* AC1, *S. pyogenes* S211 and *S. pyogenes* 02C1064 were used as positive PCR controls for the *erm(B), erm(A)* and *mef(A)* genes, respectively.

**Results**

**Antibiotic activities: overall susceptibility and resistance rates**

MIC ranges, MICₖ and MIC₀ values and percentage susceptibilities to antibiotics for all *S. pyogenes* isolates are shown in Table 1. As expected, all isolates were susceptible to penicillin (MIC₀ 0.01 mg/L). An overall 23% of isolates displayed erythromycin resistance (MIC ≤ 0.5 mg/L, intermediate plus full resistance). No statistical significance (*χ²* test) was observed when comparing the number of erythromycin-resistant strains from adults (21.7%) or paediatric (24.3%) populations. In the case of ketolides, when a breakpoint for resistance of ≥ 2 mg/L was adopted for both compounds, 2.7% of isolates were resistant to telithromycin whereas no isolates resistant to cethromycin were found. The rates of clindamycin and quinupristin–dalfopristin resistance were 3.2% and 0.5%, respectively.

According to the results of the disc diffusion assays, 97.8% of isolates were susceptible to chloramphenicol whereas 89% were susceptible to tetracycline.

**Macrolide resistance genotypes: role in phenotypic antibiotic susceptibility patterns**

Double disc induction test results with erythromycin and clindamycin were negative in all erythromycin-susceptible isolates and in those isolates with *mef(A)* as the sole erythromycin resistance gene. The disc induction test was positive in the case of isolates harbouring *erm(A)* alone (n = 3) or in combination with *mef(A)* (seven out of 11 isolates, erythromycin MICs of 1–8 mg/L). Induction was undetectable when the presence of *erm(B)* gene was inferred due to the full resistance to both erythromycin (MIC range 64–128 mg/L) and clindamycin (MIC range, 32–128 mg/L).

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**Table 1. Comparative in vitro activities of ketolides against 412 *S. pyogenes* isolates**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC (mg/L)</th>
<th>50%</th>
<th>90%</th>
<th>Susceptible (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>≤0.004–0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>100</td>
</tr>
<tr>
<td>Telithromycin</td>
<td>≤0.004–0.32</td>
<td>0.03</td>
<td>0.5</td>
<td>96.1</td>
</tr>
<tr>
<td>Cethromycin</td>
<td>≤0.004–2</td>
<td>0.008</td>
<td>0.06</td>
<td>99.8</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.01–128</td>
<td>0.03</td>
<td>16</td>
<td>77.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>≤0.01–128</td>
<td>0.03</td>
<td>0.06</td>
<td>96.8</td>
</tr>
<tr>
<td>Quinupristin–dalfopristin</td>
<td>0.06–2</td>
<td>0.5</td>
<td>1</td>
<td>99.5</td>
</tr>
</tbody>
</table>

*a* 50% and 90% MICs at which 50% and 90% of isolates are inhibited, respectively.

*b* NCCLS breakpoints.

1 For telithromycin and cethromycin, a breakpoint of ≤ 1 mg/L was considered.
which also includes erythromycin intermediate isolates, the with erythromycin MICs equal to or higher than 0.5 mg/L, a value in Table 2. Figure 1 shows MIC distribution of erythromycin, studied. In 11 (2.7%) and four (1%) isolates, respectively, the detected, being present in 69 out of the total 412 isolates (16.7%) gene was the most prevalent macrolide resistance determinant.

### Table 2. Susceptibilities of *S. pyogenes* isolates according to the presence or absence of erythromycin resistance determinants

<table>
<thead>
<tr>
<th>Resistance genotype (no. of isolates) and antibiotic</th>
<th>MIC (mg/L) range</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. resistance determinants (317)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>telithromycin</td>
<td>≤0.004–0.06</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>cethromycin</td>
<td>≤0.004–0.06</td>
<td>≤0.004</td>
<td>0.008</td>
</tr>
<tr>
<td>erythromycin</td>
<td>≤0.01–0.25</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>clindamycin</td>
<td>≤0.01–0.5</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>quinupristin–dalfopristin</td>
<td>0.12–2</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

**mef(A) (69)**

| telithromycin                                       | 0.03–2           | 0.5 | 1   |
| cethromycin                                         | ≤0.004–0.12      | 0.06| 0.06|
| erythromycin                                        | 1–16             | 8   | 16  |
| clindamycin                                         | ≤0.01–0.06       | 0.03| 0.06|
| quinupristin–dalfopristin                           | 0.06–1           | 0.5 | 1   |

**mef(A) and erm(A) (11)**

| telithromycin                                       | 0.01–1           | 0.25| 1   |
| cethromycin                                         | ≤0.004–0.12      | 0.03| 0.06|
| erythromycin                                        | 1–16             | 4   | 16  |
| clindamycin                                         | ≤0.01–0.06       | 0.03| 0.06|
| quinupristin–dalfopristin                           | 0.12–1           | 0.25| 1   |

**erm(B) (8)**

| telithromycin                                       | 2–32             |     |     |
| cethromycin                                         | 0.01–1           |     |     |
| erythromycin                                        | 64–128           |     |     |
| clindamycin                                         | 32–128           |     |     |
| quinupristin–dalfopristin                           | 0.12–1           |     |     |

**mef(A) and erm(B) (4)**

| telithromycin                                       | 8–32             |     |     |
| cethromycin                                         | 0.12–2           |     |     |
| erythromycin                                        | 128              |     |     |
| clindamycin                                         | 128              |     |     |
| quinupristin–dalfopristin                           | 0.25–1           |     |     |

**erm(A) (3)**

| telithromycin                                       | 0.008–0.03       |     |     |
| cethromycin                                         | ≤0.004–0.008     |     |     |
| erythromycin                                        | 0.5              |     |     |
| clindamycin                                         | 0.03–0.12        |     |     |
| quinupristin–dalfopristin                           | 0.12–1           |     |     |

The corresponding antibiotic susceptibilities of isolates, either with or without macrolide-resistance genetic determinants, are reported in Table 2. Figure 1 shows MIC distribution of erythromycin, telithromycin, cethromycin and clindamycin, respectively, considering the presence of different macrolide-resistance markers.

When PCR experiments were carried out in *S. pyogenes* isolates with erythromycin MICs equal to or higher than 0.5 mg/L, a value which also includes erythromycin intermediate isolates, the *mef(A)* gene was the most prevalent macrolide resistance determinant detected, being present in 69 out of the total 412 isolates (16.7%) studied. In 11 (2.7%) and four (1%) isolates, respectively, the *erm(A)* and *erm(B)* determinants were simultaneously detected with the *mef(A) gene*. *erm(B)* as sole resistance determinant was detected in eight isolates (1.9%) of the studied population.

The *mef(A)*-gene alone was present in isolates with erythromycin MIC values ranging from 1 to 16 mg/L. However, erythromycin MIC values were between 60- and 100-fold higher than those observed in *mef(A)-*negative isolates (MIC range ≤0.01–0.25 mg/L). Conversely, in *mef(A)-*carrying isolates, the corresponding MIC ranges for ketolides were significantly lower when compared with those of erythromycin, 0.03 to 2 mg/L for telithromycin and ≤0.004 to 0.12 mg/L for cethromycin. Nevertheless, the telithromycin MIC range was slightly displaced to higher values in *mef(A)-positive* isolates (up to 2 mg/L) when compared with *mef(A)-negative* isolates (0.25 mg/L). This displacement was less pronounced for cethromycin (from 0.06 to 0.12 mg/L). Erythromycin, telithromycin and cethromycin modal MIC values for susceptible isolates were 0.01, 0.008 and ≤0.004 mg/L, respectively, and for *mef(A)-positive* isolates, 16, 0.5 and 0.06 mg/L, respectively. All isolates with the *mef(A)* gene showed the typical M phenotype, with full susceptibility to clindamycin (MIC range ≤0.01 to 0.06 mg/L), except when *erm(B)* was also present (clindamycin MIC 128 mg/L).

The presence of *erm(B)* as sole erythromycin resistance determinant was confirmed in eight isolates and was responsible for the increase in telithromycin and cethromycin MIC values up to 32 and 1 mg/L, respectively. Ribosomal modification due to the action of Erm(B) methylase rendered, as previously documented, complete inactivation of both erythromycin and clindamycin (MICs up to 128 mg/L) assuming constitutive synthesis of the enzyme. *erm(A)* gene alone was observed in three isolates and its presence had almost no significance on ketolide MICs (highest values of 0.03 and 0.008 mg/L for telithromycin and cethromycin, respectively), although the erythromycin MIC value was slightly raised to 1 mg/L.

The simultaneous presence of the *erm(A)* determinant in *mef(A)* positive isolates (*n = 11*) did not increase the erythromycin, telithromycin, or cethromycin MIC values compared with those obtained in isolates with *mef(A)* gene alone (Table 2). On the contrary, the presence of *erm(B) gene* in *mef(A)* positive isolates (*n = 4*) increased erythromycin, telithromycin, and cethromycin MICs up to 128, 32 and 2 mg/L, respectively. Interestingly, the *erm(A)* gene was significantly more frequent among *mef(A)-positive* isolates (13.1%) than among *mef(A)-negative* *S. pyogenes* (0.9%), but this difference was less clear in the case of the *erm(B) gene* (4.8 versus 2.4%).

Remarkably, quinupristin–dalfopristin retained complete activity in 99.5% of isolates, despite the presence of macrolide resistance genes. It is of note that two strains exhibited quinupristin–dalfopristin MICs of 2 mg/L despite the absence of any of the studied resistance markers.

### Discussion

Telithromycin has been recently introduced into clinical practice and cethromycin is now in Phase III clinical trials. For both compounds, the main therapeutic indication is the treatment of bacterial upper and lower respiratory tract infections. The high in vitro potency demonstrated by these compounds and their refractoriness to most of the resistance mechanisms affecting macrolides suggest that ketolides should be considered as an option for the treatment of these processes where mainly streptococci (*Streptococcus pneumoniae* and *S. pyogenes*) and other Gram-positive aerobic microorganisms, *Haemophilus influenzae* and *Moraxella catarrhalis*, as well as intracellular and atypical pathogens (*Chlamydia pneumoniae*, *Legionella pneurnophila* and *Mycoplasma pneumoniae*) are most
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Macrolide resistance, particularly in the case of S. pyogenes, has been described as a fluctuating trend linked to the rise or reduction in antibiotic consumption and/or to the spread of a few predominant clones. The impact of in vitro macrolide resistance on patient outcome is controversial, particularly in the case of less severe upper respiratory tract infections like uncomplicated pharyngitis, in which an unsuccessful outcome is rare. However, certain unfavourable bacteriological courses, with persistence or relapse have been reported. In any case, absence of bacteriological response can be expected in the case of high macrolide resistance. In the case of low-level resistance, the outcome should depend on the drug, as semi-synthetic derivatives like clarithromycin and azithromycin exhibit improved antibacterial activity and pharmacodynamic properties.

In our study, when the presence of macrolide resistance mechanisms was assessed, more than 90% of the mef(A)-positive [not associated with erm(A)] S. pyogenes isolates were inhibited by telithromycin concentrations equal to or lower than 1 mg/L, an antibiotic level reached in tonsillar tissue with a normal dosage. For cethromycin, the impact of Me(A)-mediated efflux was even lower, rendering an MIC of 0.06 mg/L. The corresponding MIC values for erythromycin ranged from 1 to 16 mg/L. These results emphasize that ketolides are affected by the Me(A) efflux pump but to a much lesser extent than erythromycin. In the case of all isolates harbouring the erm(B) determinant, either alone or in combination with mef(A), telithromycin MIC values increased to resistance levels (up to 32 mg/L in two isolates), whereas cethromycin retained susceptibility levels with the exception of one isolate (MIC value of 2 mg/L). Erythromycin and clindamycin were completely inactive against these isolates (MICs up to 128 mg/L for both) strengthening the assumption of a constitutive expression of the methylase. Both ketolides were very active against strains with erm(A) harboured as sole macrolide resistance gene (MIC ranges of 0.008–0.03 mg/L and ≤0.004–0.008 mg/L for telithromycin and cethromycin, respectively) or together with mef(A), where the highest MIC values were 1 mg/L for telithromycin and 0.12 mg/L for cethromycin. These latter values were, therefore, quite similar to those observed against isolates with efflux only (Table 2). Our data may suggest that the strains harbouring mef(A) could be preferential recipients for the erm(A) gene, and may eventually be able to express high-level resistance upon induction. We could hypothesize that under conditions of macrolide challenge, S. pyogenes populations with mef(A) gene might be co-selected in the pharynx with different erm(A) potential donors, e.g. Peptostreptococcus spp. thus facilitating horizontal gene transfer. Given that ketolides are able to reduce mef(A) harbour-
ing populations, this genetic trend might be expected to be minimized if ketolides are used in the clinical setting.

Recently described ribosomal mutations (23S rRNA or L4 ribosomal protein) in \textit{S. pyogenes} were not investigated in this study; nevertheless, they do not seem to affect the activity of ketolides.\textsuperscript{14,15}

Although \textit{S. pyogenes} remains universally susceptible to penicillin, clinical treatment failures (recurrent pharyngitis) with this antibiotic occur and can be attributed to multifactorial causes. Among these can be cited poor patient compliance, insufficient period of treatment, immunological defects, lack of bacterial interference, antibiotic inactivation due to β-lactamase-producing organisms that cohabit the nasopharynx and bacterial persistence in respiratory epithelial cells where β-lactams are not capable of entering.\textsuperscript{31-33} It is of note that, if the concomitant macrolide resistance trend continues towards higher rates or even maintains its present levels, and considering the preclusion of quinolone use in the paediatric population, an alternative treatment would be needed, particularly in those geographical areas with a high incidence of macrolide resistance. In summary, ketolides display a good \textit{in vitro} activity against \textit{S. pyogenes} isolates. However, more extensive information about clinical efficacy is still awaited thus ascribing them a role in clinical practice.

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