Evolution of erythromycin resistance in *Streptococcus pneumoniae* in Italy

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**Objectives**: To evaluate erythromycin resistance in recent invasive isolates of *Streptococcus pneumoniae* in Italy, to study the phenotypic and genotypic characteristics of the isolates, and to compare data with those obtained in a previous survey.

**Methods**: Invasive pneumococcal isolates were obtained from 56 laboratories throughout the country, in 2001–2003. Isolates were serotyped and antimicrobial susceptibilities determined by Sensititre panels and Etest. A new PCR was performed to detect erythromycin resistance genes. Typing methods for selected erythromycin-resistant isolates included PFGE and multilocus sequence typing (MLST).

**Results**: One hundred and fifty-five isolates out of 444 (34.9%) were resistant to erythromycin: 95 isolates (21.4%) carried *erm*(B), 56 (12.6%) carried *mef*(A) and three carried both genes. One isolate, carrying neither *erm*(B) nor *mef*(A), showed a point mutation in domain V of the 23S rRNA genes. The *mef*(A)-positive isolates carried subtype *mef*(A) (47 isolates), subtype *mef*(E) (nine isolates), and both subtype *mef*(E) and *erm*(B) (three isolates). All subtype *mef*(A) strains, except two, belonged to serotype 14, appeared to be clonally related by PFGE and related to the England 14-9 clone by MLST. The two isolates belonging to other serotypes showed different genetic backgrounds.

**Conclusions**: Erythromycin resistance in *S. pneumoniae* has increased in the last few years in Italy. *erm*(B) is still the predominant resistance determinant; however, the increase in erythromycin resistance (34.9% versus 28.8% of the previous years) is mainly due to an increase in the proportion of isolates carrying the efflux pump *mef*(A), whereas the proportion of isolates carrying *erm*(B) has not changed.

Keywords: macrolides, molecular typing, *erm*(B), *mef*(A)

**Introduction**

In recent years, resistance to macrolides in *Streptococcus pneumoniae* has increased in many parts of the world, including Italy.1 Besides the well-known resistance determinants—*erm*(B), conferring the MLSB phenotype and *mef*(A), conferring the M phenotype—new mechanisms involving mutations of ribosomal proteins or 23S rRNA genes have been described.2 The *mef*(A) gene comprises two variants, *mef*(A) and *mef*(E), which are 90% identical at the nucleotide level, and are carried by different genetic elements, Tn1207.1 and mega, respectively.3,4 We and others have chosen to examine the two variant genes separately, due to differences in the properties of the genetic elements, and therefore we will refer to them as subtype *mef*(A) and subtype *mef*(E), respectively.5,6

In a previous study, on a large collection of *S. pneumoniae* strains from invasive pneumococcal disease (IPD) isolated in Italy in the years 1997–2000, the resistance rate to erythromycin was found to be 28.8%,7 one of the highest in Europe.1 The majority of the erythromycin-resistant isolates showed an MLSB phenotype and carried *erm*(B), whereas one quarter of the resistant isolates carried *mef*(A), commonly subtype *mef*(A).
Erythromycin resistance in *Streptococcus pneumoniae*

The aim of the present study was to evaluate the evolution of erythromycin resistance in *S. pneumoniae* in Italy in terms of resistance phenotypes and genetic determinants.

**Materials and methods**

**Bacterial isolates**

*S. pneumoniae* isolates from blood or CSF were obtained from 56 Italian hospital laboratories as part of the National Surveillance of Bacterial Meningitis (http://www.simiss.it/meningite_batterica.htm) and the AR-ISS Surveillance (http://www.simiss.it/antibiotico_resistenza.htm). Identification of the pneumococcal isolates was confirmed by conventional techniques and serotyping was performed using the antisera produced by the Statens Serum Institut (Copenhagen, Denmark). The control strains previously characterized and used for the multiplex PCR were: PN99 [erm(B)],8 PN137 [subtype mef(A)],9 PGX1416 [both erm(B) and subtype mef(E)], obtained from Glaxo, Verona, Italy, and ATCC 49619 (erythromycin susceptible, negative control).

**Antimicrobial susceptibility tests**

Determination of MICs to penicillin, erythromycin, clindamycin, tetracycline and chloramphenicol was performed by a microdilution method (Sensititre panel, Biomedical s.r.l., Scorzé, Venice, Italy) and when necessary was confirmed by Etest. The NCCLS breakpoints were applied for the interpretation of the results.9

**Detection of erythromycin resistance genes**

A multiplex PCR was developed for the simultaneous detection of *erm* (B) and of subtype *mef* (A) and subtype *mef* (E) in one reaction. Five oligonucleotide primers were used: EB1 and EB2 to detect *erm* (B),10 OM10 (5′-AGCATTGGAACAGCCTTTCA-3′), a forward primer designed in a consensus sequence of the *mef* (A), and two reverse primers, MEFA (5′-ATTTTGCCGTAGTACAGGC-3′) and MEFE (5′-TACATGGTTTCCGAAGGC-3′) to detect subtype *mef* (A) and subtype *mef* (E), respectively. The assay was based on a previously described duplex PCR10 with the following modifications: concentration of MgCl$_2$ was 4.5 mM, concentration of EB1 and EB2, 1 µM each, concentration of OM10, MEFA and MEFE, 0.5 µM each. The expected sizes of the PCR products were 639, 519 and 318 bp for *erm* (B), subtype *mef* (E) and subtype *mef* (A), respectively. Positive and negative controls were run concurrently with the isolates under study.

To detect mutations in the ribosomal proteins L4 and L22 or in the domain V of each of the four 23S rRNA alleles, amplicons were obtained and sequenced according to published methods.2,11

**Molecular typing of the isolates**

Relatedness among selected strains carrying *mef* (A) was studied by PFGE, as described previously,1 comparing visually the macrorestriction profiles obtained following *Smal* digestion.

MLST was performed on representative isolates following the recommended procedure.12

**Results and discussion**

In the years 2001–2003, 444 *S. pneumoniae* isolates (excluding duplicate isolates from the same patient) were obtained from 56 hospital laboratories around Italy. Of these isolates, 164 (37%) were from CSF and 280 (63%) from blood.

Forty-five (10.1%) strains were non-susceptible to penicillin; of these, 22 (5%) isolates were fully resistant (MIC range 2–4 mg/L) and 23 (5.1%) intermediately resistant to penicillin. This rate is similar to the rate obtained in isolates from the years 1997–2000 (9.9%).1

One-hundred and fifty-five isolates (34.9%) were resistant to erythromycin: 99 isolates (22.3%) showed high-level resistance (MIC $\geq$ 256 mg/L) to erythromycin and resistance to clindamycin (MLS$_B$ phenotype), and 56 strains (12.6%) showed varying levels of erythromycin resistance (MIC range 4–128 mg/L) and susceptibility to clindamycin (M phenotype). Among the MLS$_B$ phenotype isolates, penicillin, tetracycline and chloramphenicol resistance rates were 30%, 89.4% and 23.5%, respectively. The M phenotype isolates, with one exception (a single tetracycline resistant isolate), exhibited susceptibility to penicillin, tetracycline and chloramphenicol.

By the multiplex PCR, products of the expected size for each of the five positive controls were obtained, whereas no amplicon was yielded by the negative control. After examining a sample of isolates by both the previous method9 and the multiplex PCR, this assay was applied to all the isolates under study. Among the 99 strains showing the MLS$_B$ phenotype, 95 isolates carried *erm* (B), three isolates *erm* (B) in association with subtype *mef* (E), whereas in one isolate neither *erm* (B) nor *mef* (A) was detected. All the 56 isolates showing an M phenotype carried *mef* (A); in particular 47 isolates (84%) carried subtype *mef* (A) and nine (16%) subtype *mef* (E).

Table 1 shows the comparison between the rate of erythromycin resistance and the frequency of different resistance genotypes in the isolates examined in this study and in a previous study in the years 1997–2000, as part of similar surveillance systems.2 Interestingly, the increase in erythromycin resistance in 2001–2003 appears to be due to an increase in the percentage of isolates carrying *mef* (A), whereas the percentage of isolates carrying *erm* (B) appears unchanged over the years. Within *mef* (A), the relative percentages of the two subtypes, *mef* (A) and *mef* (E), have remained unchanged (not shown). Moreover, an increase in the MIC conferred by the efflux mechanism has been noted, up to 128 mg/L, a level we had not observed before using the same method (Etest).

There was also a new finding in that three isolates carried both *erm* (B) and subtype *mef* (E), a genotype not found previously in Italian invasive isolates. This is in accordance with Farrell et al.,5 who found that subtype *mef* (E) but not subtype *mef* (A), was associated with *erm* (B) in a large series of double-gene isolates from different geographical areas.

In one erythromycin-resistant isolate showing an erythromycin MIC $\geq$ 256 mg/L and a clindamycin MIC = 4 mg/L, neither *erm* (B) nor *mef* (A) were detected. In an experimental microarray designed to detect macrolide resistance genes, this isolate did not appear to contain any of the known erythromycin resistance determinants (M. Cassone, Università degli Studi di Siena, personal communication). No mutations were detected in the relevant regions of the ribosomal proteins L4 and L22; however, the mutation A2059G was present in three 23S rRNA alleles. This is the most common mutation responsible for erythromycin resistance in *S. pneumoniae*.2,11 Although we were unable to amplify the fourth 23S rRNA allele using the published methods, the high level of erythromycin resistance of the isolate indicates
that the mutation should involve all the 23S rRNA alleles, since
the level seems to be dependent on the number of mutated
alleles.11

The erythromycin-resistant isolates belonged to a variety of
serotypes. The 95 \textit{erm}(B)-positive isolates belonged to 15 differ-
ent serotypes, the most frequent being 19F (22.1%), 14 (15.7%),
6B (13.7%), 23F (12.6%) and 15B (8.4%), whereas 45 of 47
isolates carrying subtype \textit{mef}(A) belonged to serotype 14. By
PFGE, these isolates appeared to be clonally related, as their
profiles differed by three bands or fewer (not shown). However,
no epidemiological connection was apparent as the strains were
isolated in nine different Italian regions over 3 years. The two
isolates carrying subtype \textit{mef}(A) and belonging to serotypes 6A
and 11A, respectively, showed very different PFGE profiles (not
shown), indicating different genetic backgrounds.

The nine isolates carrying subtype \textit{mef}(E) belonged to six
different serotypes. The three isolates carrying both \textit{erm}(B)
and subtype \textit{mef}(E) belonged to serotype 14 and displayed identical
or similar profiles by PFGE (not shown), indicating different genetic backgrounds.

The nine isolates carrying subtype \textit{mef}(E) belonged to six
different serotypes. The three isolates carrying both \textit{erm}(B)
and subtype \textit{mef}(E) belonged to serotype 14 and displayed identical
or similar profiles by PFGE, related to those of the serotype 14
isolates previously described (not shown).

The MLST allelic profiles obtained for some representative
isolates are shown in Table 2. Two isolates carrying subtype
\textit{mef}(A) had identical sequence type (ST9), already observed in a
similar isolate from 1999 and corresponding to the England14-9
close. This indicates that in Italy the efflux mechanism is car-
ried predominantly by a well-established penicillin-susceptible
serotype 14 clone carrying subtype \textit{mef}(A). The two isolates
belonging to serotypes 6A and 11A show different STs, ruling
out the possibility of a capsular switch. The serotype 14 isolate
carrying both \textit{erm}(B) and subtype \textit{mef}(E) belonged to ST15, a
single-locus variant of ST9, already described in an Italian sero-
type 14 strain from 1998 carrying \textit{erm}(B).8

In conclusion, an increase in erythromycin resistance in pneu-
mococcal isolates in Italy is mainly due to an increase in the
proportion of isolates carrying the efflux mechanisms. Evolution
includes the appearance of new genotypes, namely isolates car-
ying two erythromycin resistance genes or mutations in 23S
rRNA, and M phenotypes with higher levels of resistance. Clo-
nal expansion seems to account for the spreading of serotype 14
isolates carrying subtype \textit{mef}(A). However, horizontal transfer of
resistance determinants appears to play a role in the spreading of
both \textit{erm}(B) and subtype \textit{mef}(E), since isolates with different
serotypes are recruited among the resistant isolates, and genetic
determinants of resistance tend to accumulate in the same
strains.

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Table 1. Erythromycin resistance rates and resistance determinants in pneumococcal invasive isolates in Italy

<table>
<thead>
<tr>
<th>Years</th>
<th>No. of isolates</th>
<th>No. (%) of erythromycin-resistant isolates</th>
<th>No. (%) of isolates with resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997–2000</td>
<td>503</td>
<td>145 (28.8)</td>
<td>108 (21.5)</td>
</tr>
<tr>
<td>2001–2003</td>
<td>444</td>
<td>155 (34.9)</td>
<td>95 (21.4)</td>
</tr>
</tbody>
</table>

\[^{a}\text{Includes subtype \textit{mef}(E) only.}\]
\[^{b}\text{Data from reference 7.}\]
\[^{c}P < 0.05.\]
\[^{d}\text{A2059G mutation in 23S rRNA.}\]

Table 2. Characteristics and allelic profiles (MLST) of selected erythromycin-resistant \textit{S. pneumoniae} isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Year</th>
<th>Serotype</th>
<th>Resistance determinant</th>
<th>ST[^{a}]</th>
<th>aroE</th>
<th>gdh</th>
<th>gki</th>
<th>recP</th>
<th>spi</th>
<th>xpt</th>
<th>ddl</th>
</tr>
</thead>
<tbody>
<tr>
<td>PN137</td>
<td>1999</td>
<td>14</td>
<td>subtype \textit{mef}(A)</td>
<td>91</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>AP043</td>
<td>2003</td>
<td>14</td>
<td>subtype \textit{mef}(A)</td>
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<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
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<tr>
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<td>2003</td>
<td>14</td>
<td>subtype \textit{mef}(A)</td>
<td>91</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>8</td>
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<tr>
<td>AP041</td>
<td>2003</td>
<td>6A</td>
<td>subtype \textit{mef}(A)</td>
<td>1486</td>
<td>7</td>
<td>5</td>
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<td>5</td>
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<td>20</td>
<td>28</td>
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<td>AP156</td>
<td>2003</td>
<td>11A</td>
<td>subtype \textit{mef}(A)</td>
<td>62</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>12</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>PN99[^{c}]</td>
<td>1998</td>
<td>14</td>
<td>\textit{erm}(B)</td>
<td>15</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>8</td>
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<tr>
<td>PT009</td>
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<td>14</td>
<td>subtype \textit{mef}(E), \textit{erm}(B)</td>
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<td>1</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

\[^{a}\text{Sequence type.}\]
\[^{b}\text{Isolate from a previous study.}\]
\[^{c}\text{Isolate from a previous study.}\]
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References