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Prevalence of mef and ermB genes in invasive pediatric erythromycin-resistant Streptococcus pneumoniae isolates from Argentina
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Prevalence of mef and ermB genes in invasive pediatric erythromycin-resistant Streptococcus pneumoniae isolates from Argentina


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ABSTRACT

During the period 1993-2001, a total of 1,499 pneumococci isolates were recovered through the Argentinean surveillance of Streptococcus pneumoniae causing invasive disease in children under 6 years of age, 3.5% of which were erythromycin resistant. Among the 50 erythromycin-resistant strains available, 58% (n=29) harbored mef/E genes (15 mefA, 30%; and 14 mefE, 28%), 34% (n=17) ermB, and 6% (n=3) both mefAE plus ermB genes, while one isolate was negative for all the acquired genes studied. The England14-9 (42%), Poland46-20 (20%) and Spain9V-3 (16%) clones were responsible for the emergence of pneumococcal macrolide resistance in pediatric population from Argentina.

Key words: Streptococcus pneumoniae, macrolide, Argentina, mef, erm

RESUMEN

Prevalencia de los genes mef y ermB en aislamientos invasivos de Streptococcus pneumoniae resistentes a eritromicina recuperados de pacientes pediátricos en Argentina. En el marco del programa de vigilancia regional SIREVA, se analizaron 1499 aislamientos de Streptococcus pneumoniae causantes de enfermedad invasiva en menores de 6 años, recuperados entre 1993 y 2001. Se detectó un 3.5% de resistencia a eritromicina. De los 50 aislamientos resistentes a eritromicina que pudieron ser estudiados, el 58% (n=29) tenían los genes mef/E (15 mefA, 30% y 14 mefE, 28%), el 34% (n=17) el gen ermB y el 6% (n=3) la combinación de genes mef/A/E y ermB. Sólo un aislamiento fue negativo para todos los genes analizados. Los clones internacionales England14-9, Poland46-20 y Spain9V-3 representaron el 78% del total de aislamientos resistentes (42, 20 y 16%, respectivamente) y se consideraron los responsables de la emergencia de la resistencia a macrólidos entre los neumocos que afectan a la población pediátrica de Argentina.

Palabras clave: Streptococcus pneumoniae, macrólidos, Argentina, mef, erm

INTRODUCTION

Streptococcus pneumoniae is an important cause of morbidity and mortality in humans and one of the major causes of meningitis, community-acquired pneumonia and other respiratory tract infections (19). Over the last years, resistance to penicillin G in S. pneumoniae has increased in Latin America and worldwide (6, 11, 13). Consequently, macrolides are being used as initial empiric therapy for community-acquired respiratory tract infections (12). Unfortunately, resistance to macrolides in pneumococci has also evolved rapidly (3, 6, 9, 13, 15, 17) and a total of four different mechanisms of resistance have been described: i) antibiotic efflux, mediated by mef/E genes, ii) target-site modification by methylation of 23S rRNA due to erm genes, iii) mutations in domain II or V of 23S rRNA, and iv) mutations in riboproteins L4 or L22 (12, 17). The mef/A/
E genes have been associated with erythromycin resistance (14- and 15-membered macrolides) and display an M-phenotype (14, 17). The ermB gene is known to confer cross-resistance to macrolides, lincosamides and streptogramins B, yielding the MLSB-phenotype (12).

Since 1993, Argentina and other Latin American countries have participated in the Sistema Regional de Vacunas (SIREVA) Project, a regional epidemiological surveillance conducted by the Pan American Health Organization (11). The SIREVA Project focuses on the prevalence of capsular types and antimicrobial resistance patterns of S. pneumoniae causing invasive infections in children under 6 years of age. During the period 1993-2001, a total of 1,499 invasive S. pneumoniae isolates were collected in Argentina through the SIREVA surveillance, 52 of which were erythromycin-resistant. Macrolide resistance emerged in Argentina in 1995 and increased from 0% in the period 1993-1994 to 1.4% in 1995-1997 and 6% in 1998-2001.

The aim of the present study was to determine both the prevalence of mefA/E and erm genes, and the clonal relationship among invasive erythromycin-resistant S. pneumoniae isolates.

MATERIALS AND METHODS

A total of 1,499 S. pneumoniae were submitted to the National Reference Laboratory from 39 hospitals, 17 provinces and Buenos Aires city. Fifty out of 52 erythromycin-resistant isolates from 14 hospitals (8 cities, 7 provinces) were available for further investigation. Thirty-one isolates (62%) were recovered from patients with pneumonia, twelve (24%) with meningitis, and seven (14%) with other infections. This subset of resistant isolates was recovered from children under 6 years old (0-6 months, 14 strains; 6-12 months, 12 strains; 1 year, 12 strains; and 2 years, 2 strains), the remaining 10 strains were from children ranging from >2 to <6 years old. Serotyping was determined by the capsular Quellung method using commercial antisera (Statens Seruminstitut, Copenhagen, Denmark) as recommended by the manufacturer. Minimal inhibitory concentrations (MICs) were determined by the agar dilution procedure using Mueller-Hinton agar (Difco, BD, USA) supplemented with 5% horse blood and incubated overnight at 35 °C. MICs values to penicillin, cefotaxime, trimethoprim-sulfamethoxazole, tetracycline, chloramphenicol, ofloxacin, vancomycin, erythromycin, azithromycin, and clindamycin were interpreted according to CLSI guidelines (7). Isolates showing resistance to erythromycin but susceptibility to clindamycin expressed the M-phenotype, while isolates showing resistance to both erythromycin and clindamycin were grouped as having the MLSB-phenotype.

Double disk diffusion assays using erythromycin (15 µg) and clindamycin (2 µg) disks (disk supplied by BBL, BD, USA) were performed in order to evaluate the inducible or constitutive expression of the MLSB-phenotype.

DNA templates were prepared by the boiling method and 5 µl of these extracts were used in each PCR assay. Reactions were performed under standard conditions in a final volume of 50 µl, using a Biometra thermal cycler (Whatman Biometra GmbH, Göttingen, Germany). Amplification of mefA/E, ermB, ermC, ermA and ermTR genes was performed with previously reported primers (24).

Discrimination between mefA and mefE alleles was performed by BamHI (New England Biolabs, Beverly, MA, USA) cleavage of the 348-bp mefA/E amplicon (17). Briefly, BamHI renders two fragments of 284 and 64 bp with the mefA allele, while mefE has no restriction sites.

PFGE was carried out using Smal (New England Biolabs, Beverly, MA, USA) as previously described (8). DNA fragments were resolved in 1% agarose gel using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA, USA) by applying a switch time of 5 to 35 seconds during 23 h at 11.3 °C (8). PFGE patterns were visually compared to 16 international clones described by McGee (16), kindly provided by Marguerite Lovgren (National Centre for Streptococcus, Edmonton, Alberta, Canada), and categorized using the Tenover criteria (25). MLST was performed at the Microbiology Laboratory, The Rockefeller University, NY, USA as previously described (27).

RESULTS AND DISCUSSION

Among the 50 erythromycin-resistant S. pneumoniae examined in this study, 25 (50%) and 8 (16%) isolates displayed non-susceptibility to penicillin and cefotaxime, respectively. Resistance to tetracycline and chloramphenicol was observed in 22 (44%) and 6 (12%) isolates, respectively. All isolates were susceptible to ofloxacin and vancomycin (Table 1). The MIC range for erythromycin and azithromycin was 4 - ≥512 µg/ml, while the range for clindamycin was 0.06-512 µg/ml. Isolates were grouped into those expressing the M-phenotype (n=29; 58%) or the MLSB-phenotype (n=21; 42%), and no induction was observed for the strains expressing the MLSB-phenotype (Table 1). Isolates displaying the MLSB-phenotype showed higher MICs to erythromycin, azithromycin and clindamycin than isolates with the M-phenotype, and were also associated with a high percentage of resistance to penicillin, tetracycline and chloramphenicol (Table 1).

All 29 M-phenotype isolates were positive for mefA/E genes (Table 2). Among the MLSB-phenotype isolates (n=21), 17 yielded PCR positive for ermB gene and 3 contained both mefA/E and ermB genes. One isolate displaying the MLSB-phenotype was repeatedly negative for mefA/E, ermB, ermC, ermA and ermTR acquired genes. Therefore, mutation in 23S rRNA or in riboprotoins L4 or L22 might be a likely mechanism involved in this phenotype (12).

When we analyzed erythromycin resistance determinants in the complete set of 50 isolates, it was observed that 58% harbored the mefA/E genes, 34% the ermB and 6% both the mefA/E and the ermB genes. In North-American countries, the mef genes are more prevalent in S. pneumoniae (4, 12). In contrast, in Europe and South Africa, the ermB gene is the most frequent, while in Asian countries, the prevalence of genotypes is variable (12, 21, 23). Additionally, we determined by PCR-RFLP that 17 (53%) strains harbored the mefE allele, while the remaining 15 (47%), the mefA variant (Table 2). The mefA and mefE alleles are located in different genetic elements, Tn1207.1 and MEGA, respectively, showing different epi-
Table 1. Susceptibility of *S. pneumoniae* isolates according to erythromycin-resistant phenotype.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MIC range</th>
<th>ERY</th>
<th>AZM</th>
<th>CLI</th>
<th>PEN</th>
<th>CTX</th>
<th>SXT</th>
<th>TET</th>
<th>CHL</th>
<th>VAN</th>
<th>OFX</th>
</tr>
</thead>
<tbody>
<tr>
<td>M (n=29)</td>
<td>4-32</td>
<td>4-64</td>
<td>0.06-0.25</td>
<td>0.008-4</td>
<td>≤0.004-2</td>
<td>0.015-32</td>
<td>0.06-32</td>
<td>0.25-32</td>
<td>0.12-0.5</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>16</td>
<td>16</td>
<td>0.12</td>
<td>0.015</td>
<td>0.015</td>
<td>0.25</td>
<td>0.12</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>16</td>
<td>32</td>
<td>0.12</td>
<td>2</td>
<td>1</td>
<td>8</td>
<td>16</td>
<td>4</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% NS</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>20.7</td>
<td>13.8</td>
<td>58.6</td>
<td>13.8</td>
<td>6.9</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MLSB (n=21)</td>
<td>16-512</td>
<td>8-512</td>
<td>8-512</td>
<td>0.008-4</td>
<td>≤0.04-2</td>
<td>0.25-16</td>
<td>0.06-32</td>
<td>1-32</td>
<td>0.12-0.5</td>
<td>1-2</td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>512</td>
<td>512</td>
<td>256</td>
<td>0.12</td>
<td>0.03</td>
<td>1</td>
<td>32</td>
<td>2</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;S&lt;/sub&gt;</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>512</td>
<td>1</td>
<td>1</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>0.25</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>% NS</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>90.5</td>
<td>19</td>
<td>61.9</td>
<td>32</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

ERY, erythromycin; AZM, azithromycin; CLI, clindamycin; PEN, penicillin; CTX, cefotaxime; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; CHL, chloramphenicol; VAN, vancomycin; OFX, ofloxacine. MIC values are in µg/ml; % NS, percentage of non-susceptible strains.

Table 2. Genotypic characterization and clonal relationship of erythromycin-resistant *S. pneumoniae* isolates.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>Sma I-PFGE/International clone</th>
<th>Serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>M (29)</td>
<td>mefA (15)</td>
<td>A / England&lt;sup&gt;1&lt;/sup&gt;-9 (12)</td>
<td>14 (11), 23A (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B / Poland&lt;sup&gt;4&lt;/sup&gt;-20 (1)</td>
<td>6B (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C / Spain&lt;sup&gt;v&lt;/sup&gt;-3 (1)</td>
<td>14 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H (1)</td>
<td>19F (1)</td>
</tr>
<tr>
<td>mefE (14)</td>
<td></td>
<td>A / England&lt;sup&gt;1&lt;/sup&gt;-9 (7)</td>
<td>14 (6), 19A (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C / Spain&lt;sup&gt;v&lt;/sup&gt;-3 (6)</td>
<td>14 (3), 9V (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G (1)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>MLSB (21)</td>
<td>ermB (17)</td>
<td>B / Poland&lt;sup&gt;4&lt;/sup&gt;-20 (9)</td>
<td>6B (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D / Spain&lt;sup&gt;6&lt;/sup&gt;-2 (3)</td>
<td>6B (2), 19F (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E / Spain&lt;sup&gt;12&lt;/sup&gt;-1 (1)</td>
<td>23F (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F (3)</td>
<td>6B (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>I (1)</td>
<td>11A (1)</td>
</tr>
<tr>
<td>ermB + mefE (3)</td>
<td></td>
<td>A / England&lt;sup&gt;1&lt;/sup&gt;-9 (2)</td>
<td>6B (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E / Spain&lt;sup&gt;12&lt;/sup&gt;-1 (1)</td>
<td>23F (1)</td>
</tr>
<tr>
<td>non gene detected (1)&lt;sup&gt;*&lt;/sup&gt;</td>
<td></td>
<td>C / Spain&lt;sup&gt;v&lt;/sup&gt;-3 (1)</td>
<td>9V (1)</td>
</tr>
</tbody>
</table>

Number of isolates are indicated in parentheses; <sup>*</sup>PCR were negative for *ermB, ermC, ermA* and *ermTR* genes.

demiological behavior as described (14, 17). Tetracycline resistance in *S. pneumoniae* displaying the MLS<sub>2</sub>-phenotype has been associated with the presence of Tn1545, Tn1116, Tn3872 or Tn6002 transposons which encode tetM and ermB genes (5, 18). Although we did not look for presence of the tetM gene, 90% (n=18) of the *S. pneumoniae* isolates harboring the ermB gene were tetracycline-resistant, which suggests the presence of Tn1545 or Tn1545-like in Argentina (data not shown).

Serotype analysis (n; %) showed that the most frequent serotypes were: 14 (22; 44%) and 6B (17; 34%), while the remaining 11 *S. pneumoniae* expressed 6 different serotypes: 9V (4; 8%), 23F (2; 4%), 19F (2; 4%), 23A (1; 2%), 11F (1; 2%) and 19F (1; 2%) (Table 2). All 22 serotype 14 isolates expressed the M-phenotype, while 16 out of 17 serotype 6B strains harbored the ermB gene (MLS<sub>B</sub>-phenotype), two of which also contained the mefE gene (Table 2). We have previously reported that *S. pneumoniae* serotype 14 was the most frequent one causing invasive infections in pediatric patients (11, 22). Herein, we found that the emergence of erythromycin-resistant *S. pneumoniae* was mainly related to both serotypes 14 and 6B.

When analyzing the SmaI-PFGE pattern, 9 clonal types (A to I) were established (Figure 1, Table 2). Moreover, 88% of all isolates were related to 5 international clones (n; %): England<sup>1</sup>-9 (21; 42%), Poland<sup>4</sup>-20 (10; 20%), Spain<sup>v</sup>-3 (8; 16%), Spain<sup>6</sup>-2 (3; 6%) and Spain<sup>12</sup>-1 (2; 4%) (Table 2). Six strains, belonging to F-I patterns, were not related to any of the international clones used in this study as described by McGee et al. (Figure 1) (16). Eighty-one percent (n=17) of the England<sup>1</sup>-9 clones were sero-
type 14, while all 10 Poland 6B-20 isolates were serotype 6B (Table 2). Erythromycin-resistant Spain 9V-3 isolates expressed serotypes 9V and 14 (Table 2). We and other authors have previously reported that the major penicillin-resistant S. pneumoniae clone in Argentina was the Spain 9V-3 serotype 14 clone (1, 22). Additionally, MLST analysis of S. pneumoniae isolates from Brazil and Uruguay also described the association of the Spain 9V-3 clone with serotype 14 (16, 20). Therefore, the presence of the Spain 9V-3 clone expressing serotype 14 seems common in our region and suggests a capsular switching of 9V to 14. A genetic analysis between Spain 9V-3 clones expressing 9V or 14 serotypes should be necessary to evaluate the origin and evolution of these clonal variants in Argentina. A representative isolate from the three major dominant clones was selected and typing was confirmed by MLST: England 14-9 (ST 9), Poland 6B-20 (ST 315) and Spain 9V-3 (ST 156). In agreement with other authors, these three international clones were mainly implicated in the global dissemination of macrolide-resistance (10, 20, 26). Nineteen out of twenty-one England 14-9 isolates harbored the \textit{mef}A/E gene (12 \textit{mef}A and 7 \textit{mef}E), and 9 out of 10 Poland 6B-20 strains carried the \textit{erm}B gene in agreement with previous data (16, 20). Association was not found between serotypes or \textit{mef} variants and geographical regions. The Spain 9V-3 clone is usually susceptible to macrolides, but in this study and, in agreement with other authors, we found an association between this clone and the \textit{mef} genes (2, 16).

In summary, 58% of the erythromycin-resistant isolates from the period 1993-2001 harbored the \textit{mef}A/E gene, 34% the \textit{erm}B gene, 6% contained the dual efflux (\textit{mef}A/\textit{E} genes) plus methylase (\textit{erm}B) mechanisms, and one isolate had neither gene. The emergence of erythromycin-resistant \textit{S. pneumoniae} was principally due to the England 14-9, Poland 6B-20 and Spain 9V-3 international clones.

Data from the Argentinean SIREVA surveillance of the period 2005-2006 showed that macrolide resistance reached 16.4% with a similar distribution of M- (57.9%) and MLSB- (42.1%) phenotypes (unpublished data). Moreover, almost 80% of these erythromycin-resistant \textit{S. pneumoniae} belong to serotypes 14 (52%) or 6B (27%) (unpublished data), which suggests that nowadays both England 14-9 and Poland 6B-20 international clones are still the major clones involved in the spread of macrolide-resistance in Argentina. This is the first study both assessing the mechanisms of macrolide resistance in invasive pediatric \textit{S. pneumoniae} isolates from Argentina, and the genetic relatedness between these organisms. Knowledge of the mechanisms and epidemiology of macrolide resistance in this pathogen is essential to predict the current and future use of these drugs as both first and second line antibiotics.
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