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Prevalence of *mef* and *erm*B 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*A/E genes (15 *mef*A, 30%; and 14 *mef*E, 28%), 34% (n=17) *erm*B, and 6% (n=3) both *mef*A/E plus *erm*B genes, while one isolate was negative for all the acquired genes studied. The England^{14}-9 (42%), Poland^{6B}-20 (20%) and Spain^{9V}-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 *erm*B 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*A/E (15 *mef*A, 30% y 14 *mef* E, 28%), el 34% (n=17) el gen *erm*B y el 6% (n=3) la combinación de genes *mef*A/E y *erm*B. Sólo un aislamiento fue negativo para todos los genes analizados. Los clones internacionales England^{14}-9, Poland^{6B}-20 y Spain^{9V}-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 neumococos 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*A/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/

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E genes have been associated with erythromycin resistance (14- and 15-membered macrolides) and display an M-phenotype (14, 17). The *erm*B gene is known to confer cross - resistance to macrolides, lincosamides and streptogramins B, yielding the MLS_B-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 *mef*A/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 blood (38%), pleural fluid (38%) and cerebrospinal fluid (24%). Eighty percent of isolates (n=40) were recovered from children ≤ 2 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 ºC. 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 MLS_B-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 MLS_B-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 *mef*A/E, *erm*B, *erm*C, *erm*A and *erm*TR genes was performed with previously reported primers (24).

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

PFGE was carried out using *Sma*I (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 ºC (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 MLS_B-phenotype (n=21; 42%), and no induction was observed for the strains expressing the MLS_B-phenotype (Table 1). Isolates displaying the MLS_B-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 *mef*A/E genes (Table 2). Among the MLS_B-phenotype isolates (n=21), 17 yielded PCR positive for *erm*B gene and 3 contained both *mef*A/E and *erm*B genes. One isolate displaying the MLS_B-phenotype was repeatedly negative for *mef*A/E, *erm*B, *erm*C, *erm*A and *erm*TR acquired genes. Therefore, mutation in 23S rRNA or in riboproteins 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 *mef*A/E genes, 34% the *erm*B and 6% both the *mef*A/E and the *erm*B genes. In North-American countries, the *mef* genes are more prevalent in *S. pneumoniae* (4, 12). In contrast, in Europe and South Africa, the *erm*B 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 *mef*E allele, while the remaining 15 (47%), the *mef*A variant (Table 2). The *mef*A and *mef*E alleles are located in different genetic elements, Tn*1207.1* and MEGA, respectively, showing different epi-

Table 1. Susceptibility of *S. pneumoniae* isolates according to erythromycin-resistant phenotype.

Phenotype		ERY	AZM	CLI	PEN	CTX	SXT	TET	CHL	VAN	OFX
M (n=29)	MIC range	4-32	4-64	0.06-0.25	0.008-4	≤0.004-2	0.015->32	0.06-32	0.25->32	0.12-0.5	1-2
	MIC_{50}	16	16	0.12	0.015	0.015	0.25	0.12	2	0.25	2
	MIC_{90}	16	32	0.12	2	1	8	16	4	0.25	2
	% NS	100	100	0	20.7	13.8	58.6	13.8	6.9	0	0
MLSB(n=21)	MIC range	16->512	8->512	8-512	=0.008-4	≤0.04-2	0.25-16	0.06->32	1-32	0.12-0.5	1-2
	MIC_{50}	512	512	256	0.12	0.03	1	32	2	0.25	2
	MIC_{90}	>512	>512	512	1	1	8	32	16	0.25	2
	% NS	100	100	100	90.5	19	61.9	85.7	19	0	0

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. Genotipic characterization and clonal relationship of erythromycin-resistant *S. pneumoniae* isolates.

Phenotype	Genotype	*Sma* I-PFGE/International clone	Serotype
M (29)	*mef*A (15)	A / England^{14}-9 (12)	14 (11), 23A (1)
		B / Poland^{6B}-20 (1)	6B (1)
		C / Spain^{9V}-3 (1)	14 (1)
		H (1)	19F (1)
	*mef*E (14)	A / England^{14}-9 (7)	14 (6), 19A (1)
		C / Spain^{9V}-3 (6)	14 (3), 9V (3)
		G (1)	14 (1)
MLS_B (21)	*erm*B (17)	B / Poland^{6B}-20 (9)	6B (9)
		D / Spain^{6B}-2 (3)	6B (2), 19F (1)
		E / Spain^{23F}-1 (1)	23F (1)
		F (3)	6B (3)
		I (1)	11A (1)
	*erm*B + *mef* E (3)	A / England^{14}-9 (2)	6B (2)
		E / Spain^{23F}-1 (1)	23F (1)
	non gene detected (1)[a]	C / Spain^{9V}-3 (1)	9V (1)

Number of isolates are indicated in parentheses; [a]PCR were negative for *erm*B, *erm*C, *erm*A and *erm*TR genes.

demiological behavior as described (14, 17). Tetracycline resistance in *S. pneumoniae* displaying the MLS_B-phenotype has been associated with the presence of Tn*1545*, Tn*1116*, Tn*3872* or Tn*6002* transposons which encode *tet*M and *erm*B genes (5, 18). Although we did not look for presence of the *tet*M gene, 90% (n=18) of the *S. pneumoniae* isolates harboring the *erm*B gene were tetracycline-resistant, which suggests the presence of Tn*1545* or Tn*1545*–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 *erm*B gene (MLS_B-phenotype), two of which also contained the *mef*E 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 *Sma*I-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^{14}-9 (21; 42%), Poland^{6B}-20 (10; 20%), Spain^{9V}-3 (8; 16%), Spain^{6B}-2 (3; 6%) and Spain^{23F}-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^{14}-9 clones were sero-

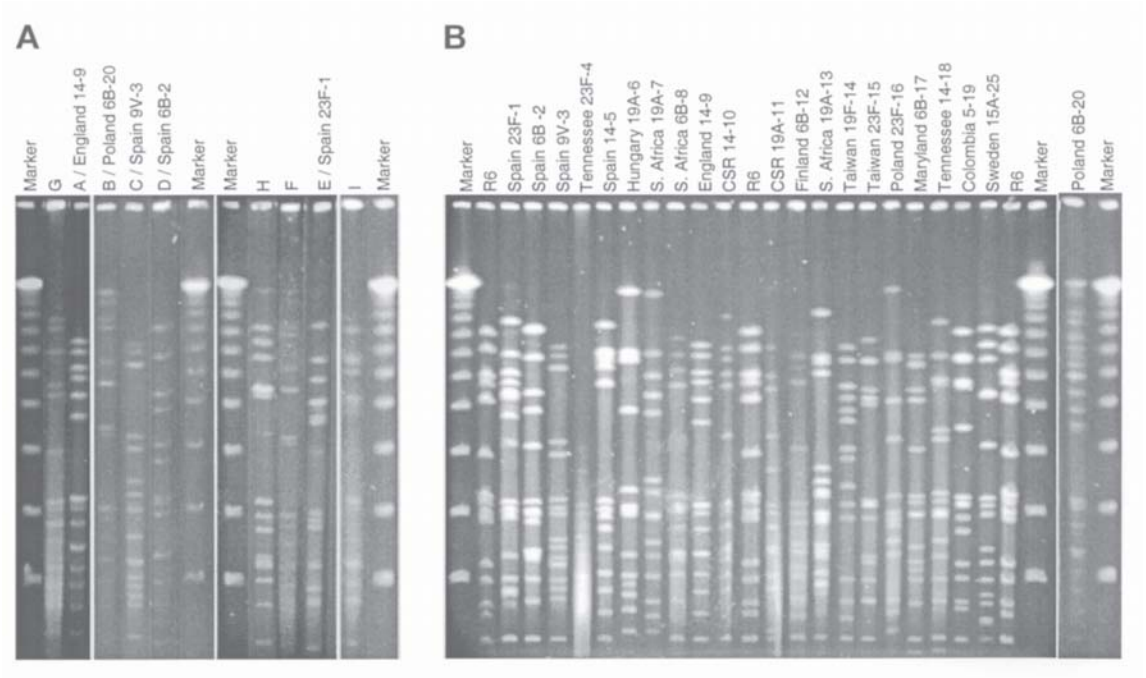

Figure 1. *Sma*I-PFGE patterns of *S. pneumoniae* isolates. Marker: Lambda ladder PFG Marker (New England Biolabs). *Sma*I-digested DNA of *S. pneumoniae* R6 was also included as reference pattern. **Panel A.** Erythromycin-resistant *S. pneumoniae* clones determined in the present study, clones A to I. Clones A to E were related to some *S. pneumoniae* international clones. **Panel B.** *S. pneumoniae* international clones described by McGee *et al.* and in PMEN web site (16, 20).

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 *mef*A/E gene (12 *mef*A and 7 *mef*E), and 9 out of 10 Poland^{6B}-20 strains carried the *erm*B gene in agreement with previous data (16, 20). Association was not found between serotypes or *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 *mef* genes (2, 16).

In summary, 58% of the erythromycin-resistant isolates from the period 1993-2001 harbored the *mef*A/E gene, 34% the *erm*B gene, 6% contained the dual efflux (*mef*A/E genes) plus methylase (*erm*B) mechanisms, and one isolate had neither gene. The emergence of erythromycin-resistant *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 MLS_B-(42.1%) phenotypes (unpublished data). Moreover, almost 80% of these erythromycin-resistant *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 *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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