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BRIEF REPORT

Macrolide resistance in *Streptococcus pneumoniae* isolated from Argentinian pediatric patients suffering from acute otitis media

Vanesa Reijtman[a], Paula Gagetti[b], Diego Faccone[b], Sofía Fossati[b], Patricia Sommerfleck[c], Claudia Hernández[a], Patricia Bernáldez[c], Horacio Lopardo[a,*], and Alejandra Corso[b]

[a] *Servicio de Microbiología, Hospital de Pediatría "Prof. Dr. J. P. Garrahan", Buenos Aires, Argentina*
[b] *Servicio Antimicrobianos, Instituto Nacional de Enfermedades Infecciosas, ANLIS "Dr. Carlos G. Malbrán", Buenos Aires, Argentina*
[c] *Servicio de Otorrinolaringología, Hospital de Pediatría "Prof. Dr. J. P. Garrahan", Buenos Aires, Argentina*

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Abstract
Macrolide-resistant *Streptococcus pneumoniae* emerged in Argentina in 1995, representing 26% of invasive infection isolates in children under 5 years old. The objectives of this study were to describe the prevalence of *ermB* and *mefA* genes in macrolide-resistant *S. pneumoniae* isolates from acute otitis media (AOM) and to determine their genetic relatedness. Between May 2009 and August 2010, 126 *S. pneumoniae* isolates from 324 otherwise healthy children with a first episode of AOM were included. Twenty six of these isolates (20.6%) were resistant to erythromycin. Most frequent serotypes were: 14 (46.2%), 6A (23.1%), 19F (7.7%) and 9V (7.7%). Twenty (76.9%) carried the *mef*A gene, 5 (19.2%) have the *erm*B gene, and 1 (3.9%) both *erm*B + *mef*A. Ten clonal types were identified, mostly related to Sweden^{15A}-25/ST782 (SLV63), CloneB^{6A}/ST473 and England^{14}-9/ST9. This is the first study assessing the mechanisms of macrolide resistance in pneumococci isolates from pediatric AOM in Argentina and their genetic relatedness.

* Corresponding author.
E-mail address: hlopar25@gmail.com (H. Lopardo).

PALABRAS CLAVE
Streptococcus pneumoniae;
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Resistencia a macrólidos en *Streptococcus pneumoniae* aislados de pacientes pediátricos con otitis media aguda en la Argentina

Resumen
Streptococcus pneumoniae resistente a macrólidos emergió en la Argentina en 1995 y representa el 26 % de los aislamientos de infecciones invasivas en niños menores de 5 años. El objetivo de este trabajo fue describir la prevalencia de *ermB* y *mefA* en neumococos resistentes a macrólidos aislados de niños con otitis media aguda (OMA) y determinar su relación genética. En 15 meses se incluyeron 126 neumococos aislados de 324 niños previamente sanos, con un primer episodio de OMA. Veintiseis (20,6 %) eran resistentes a eritromicina. Los serotipos más frecuentes fueron los siguientes: 14 (46,2 %), 6A (23,1 %), 19F (7,7 %) y 9V (7,7 %). Veinte eran portadores del gen *mef*A, 5 del gen *erm*B y el restante portaba ambos genes. Se identificaron 10 tipos clonales, la mayoría relacionados con los clones Sweden^{15A}-25/ST782 (SLV63), CloneB^{6A}/ST473 y England^{14}-9/ST9. Este es el primer estudio en determinar los mecanismos de resistencia a macrólidos en neumococos aislados de OMA en la Argentina y en describir su relación genética.

Acute otitis media (AOM) is by far the most common disease caused by *Streptococcus pneumoniae* and one of the most frequent diagnoses in children under 2 years old. One out of every three children has at least one episode of AOM during the first three years of life[1].

Over the last years, resistance to penicillin in *S. pneumoniae* has increased in Latin America and worldwide[10,11]. Consequently, macrolides were used as initial empiric therapy for community-acquired respiratory tract infections[11]. Unfortunately, resistance to macrolides in *S. pneumoniae* has also evolved rapidly and in many countries the rate of *S. pneumoniae* resistant to macrolides exceeded the level of resistance to penicillin[2,8]. Results from a global international surveillance project (PROTEKT, 1993-2003) showed an increase in macrolide resistance from 31% in 1999 to 36.3% in 2003[11]. Macrolide resistance in *S. pneumoniae* causing infectious diseases in pediatric patients emerged in Argentina in 1995 and firstly increased to 1.4% between 1995-1997 and later to 6% in the period 1998-2001[6]. Nowadays, according to the Argentinian SIREVA II surveillance data, macrolide resistance reached 30.2% in 2011[10]. Four different mechanisms of resistance to macrolides have been described: antibiotic efflux, mediated by *mefA* gene; target-site modification by methylation of 23S rRNA due to *erm* genes; mutations in riboproteins L4 or L22 and mutations in domain II or V of 23S rRNA. The *mefA* gene, displaying an M-phenotype, has been associated with resistance to 14- and 15-membered macrolides. The *ermB* gene is known to confer cross-resistance to macrolides, lincosamides and streptogramins B, yielding the MLS_B-phenotype[9].

The objectives of the present study were to determine the frequency of macrolide-resistant *S. pneumoniae* isolates from AOM, the prevalence of *ermB* (ribosomal methylase) and *mefA* (efflux pump) genes and to determine their genetic relatedness.

Between May 2009 and August 2010, 324 immunocompetent children under 120 months (median age: 8 months) with a first episode of AOM with purulent exudates in the middle ear were included in this study. Immunocompromised patients and those with chronic otitis media were excluded. All middle ear specimens obtained through tympanocentesis were cultured under standard conditions[7]. One hundred and thirty three pneumococci were isolated from 324 children suffering from AOM (41%), but 126 were available for this study.

Pneumococcal strains were serotyped by the Neufeld Quellung reaction using antiserum from the Statens Serum Institut, Copenhagen, Denmark.

Minimal inhibitory concentrations (MIC) were determined by the agar dilution method using Mueller Hinton agar (Difco, BD, USA) supplemented with 5% sheep blood and incubated overnight at 35 °C. MIC values of penicillin, amoxicillin, cefotaxime, erythromycin and clindamycin were interpreted according to Clinical and Laboratory Standards Institute recommendations. Susceptibility to chloramphenicol, tetracycline, ofloxacin, vancomycin and trimethoprim-sulfamethoxazole was tested by the disk diffusion method according to the CLSI guidelines. Phenotypic characterization of macrolide resistance was performed by the double-disk diffusion method (D test), using disks of erythromycin (15 μg) and clindamycin (2 μg) to discriminate isolates expressing the M- and MLS_B-phenotype (inducible or constitutive). MICs were interpreted according to the CLSI guidelines[5].

PCR assays for detection of *mef*A and *erm*B genes and pulse-field gel electrophoresis (PFGE) were performed as previously described (6). PFGE patterns were categorized using the Tenover criteria[14] and compared to international clones (http://www.sph.emory.edu/PMEN/). Multilocus sequence typing (MLST) was carried out as previously described, with five selected strains of each dominant PFGE pattern and those associated with international clones (www.mlst.net/).

Twenty six of the 126 available pneumococci (20.6%) were classified as erythromycin-resistant and were further studied. Although macrolide resistance in our country has been increasing steadily since 1995[10], these resistance

levels do not exceed erythromycin resistance from invasive infections in other countries[4]. Most frequent serotypes among macrolide-resistant pneumococci from AOM were (n; %): 14 (12; 46.3) and 6A (6; 23.1); followed by 19F (2; 7.7), 9V (2; 7.7), 6B (1; 3.8), 19A (1; 3.8), 33F (1; 3.8) and non-typeable (1; 3.8) compared with 14 and 6B, found in a previous study of invasive infections[6]. Almost in coincidence with our results, in Bedouin and Jewish children with AOM, predominant serotypes with macrolide resistance were: 6A, 6B, 14, 19A and 19F[13] and in Bulgarian children, the predominant serotypes were: 6B, 19A and 19F[12]. MIC range, MIC_{50} and MIC_{90} for penicillin, amoxicillin, cefotaxime, erythromycin and clindamycin of the 26 isolates are shown in table 1. In 65.4% of cases, erythromycin resistance was associated with penicillin MICs between 0.12 and 1 μg/ml. Breakpoints for penicillin were those recommended for oral penicillin V[5]. We have to emphasize that applying the current breakpoints of CLSI, all isolates were susceptible to amoxicillin, which is the antibiotic recommended for the empirical treatment of AOM in our country. All isolates were susceptible to cefotaxime (MIC ≤ 1 μg/ml), and also to chloramphenicol, ofloxacin and vancomycin when tested by the disk diffusion method, using the breakpoints recommended by the CLSI[5].

Resistance to erythromycin was recorded in 26 (20.6%) isolates of *S. pneumoniae* from AOM, mainly due to the presence of the *mef*A gene. Resistance to tetracycline and trimethoprim-sulfamethoxazole was observed in 13 (50%) and in 15 (57.7%) macrolide-resistant isolates, respectively.

Twenty of the 26 macrolide resistant isolates (76.9%) expressed the M-phenotype and were positive for the *mef*A gene, while 6 (23.1%) expressed the constitutive MLS_B-phenotype. Five of them were positive for the *erm*B gene and one was positive for both genes (table 1). As has been previously described, isolates displaying the MLS_B-phenotype showed higher MICs of erythromycin and clindamycin than those with the M-phenotype. All *S. pneumoniae* serotype 14 isolates harbored the *mef*A gene (11/12 expressed the M-phenotype and 1/12 expressed the MLS_B-phenotype due to the presence of both *mef*A and *erm*B genes). All serotype 6A isolates harbored the *mef*A gene and expressed the M-phenotype. Our results are consistent with previous Argentinian data of macrolide-resistant *S. pneumoniae* isolated between 1993 and 2001[6]. However, in European countries, macrolide-resistant *S. pneumoniae* strains are predominantly *ermB* positive[12] and in South Korea dissemination of macrolide-resistant *S. pneumoniae* was due to isolates containing the *ermB* gene alone or concomitantly with the *mefA* gene[15]. None of the macrolide-resistant isolates was found to be *mefA* and *ermB* negative. Other macrolide resistance mechanisms, such as mutations in the 23S rRNA or alterations in ribosomal proteins L4 and L22 have not been investigated.

Table 1 Minimal inhibitory concentration and correlation among phenotypes and genotypes of macrolide-resistant *Streptococcus pneumoniae*

		MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Total (26)				
M phenotype (20)				
mefA (20)	PEN	≤0.016-0.25	0.12	0.125
	AMX	≤0.016-0.25	0.06	0.125
	CTX	≤0.016-0.25	0.06	0.25
	ERY	4-64	8	64
	CLI	0.12-0.25	0.25	0.25
MLSB phenotype (6)				
ermB (5)				
ermB + *mefA* (1)	PEN	0.016-0.25	NA	NA
	AMX	≤0.016-0.125	NA	NA
	CTX	0.016-0.125	NA	NA
	ERY	1024->1024	NA	NA
	CLI	512	NA	NA

PEN, penicillin; AMX, amoxicillin; CTX, cefotaxime; ERY, erythromycin; CLI, clindamycin; NA, not applicable.

Table 2 Comparison of macrolide-resistant isolates with international clones

Clone (PFGE)	International clone by PFGE	ST	No. of isolates (%)	Serotype	Genotype
A	Sweden[15A]-25	782	7 (27)	14	*mefA* (n= 6)
					mefA+*ermB* (n=1)
B		473	6 (23.1)	6A	*mefA*
C	England[14]-9	9	4 (15.5)	14	*mefA*
D	Spain [9V]-3	162	2 (7.7)	9V	*mefA*
E			2 (7.7)	19F	*ermB*
F			1 (3.8)	33F	*ermB*
G			1 (3.8)	NT	*ermB*
H			1 (3.8)	19A	*mefA*
I	Poland[6B]-20	315	1 (3.8)	6B	*ermB*
J			1 (3.8)	14	*mefA*

NT, non-typeable.

Ten (10) clonal types (A to J) were defined by the *Sma*I-PFGE pattern. (fig.1). Seventeen isolates were grouped into three clones: clone A n:7, clone B n:6, and clone C n:4. The PFGE pattern of clones A and C were related to Sweden[15A]-25 and England[14]-9 international clones defined in PMEN while clone B[6A] was not related to any international clone. The remaining nine isolates were grouped into seven clones, of which only two were related to international clones: Spain[9V]-3 and Poland[6B]-20 (table 2). In the study of Corso *et al*. referring to invasive infections, the emergency of erythromycin-resistant *S. pneumoniae* was principally

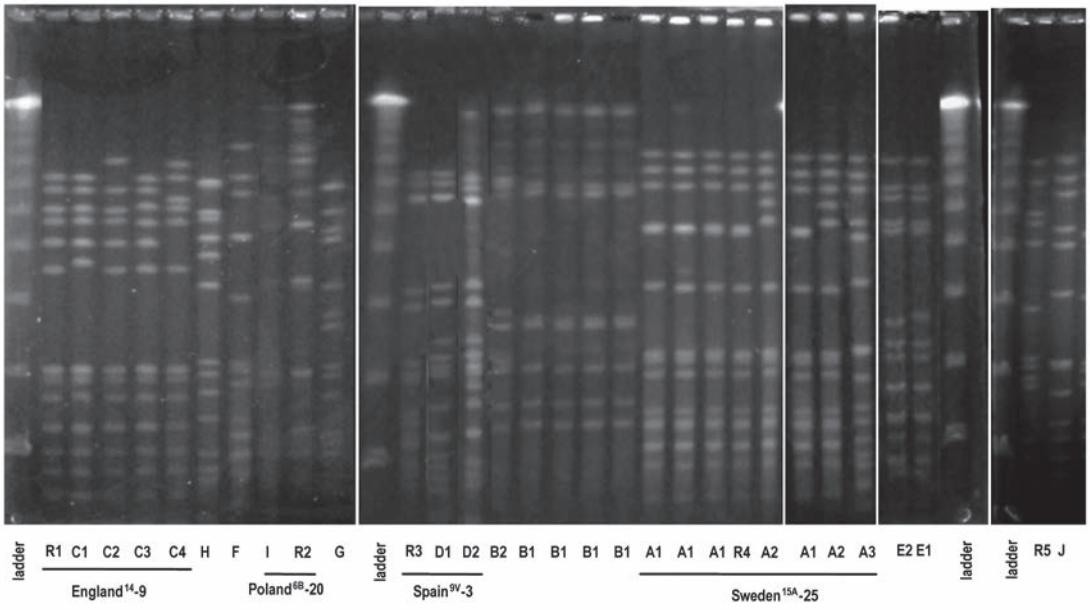

Figure 1 Pulse-field gel electrophoresis profiles of 25 erythromycin-resistant isolates and representative strains of international disseminated clones. Λ ladder, molecular size marker; R1, England^{14}-9; R2, Poland^{6B}-20; R3, Spain^{9V}-3; R4, Sweden^{15A}-25; R5, reference strain R6.

due to the England^{14}-9, Poland^{6B}-20 and Spain^{9V}-3 international clones [6], while in the present study both the Sweden^{15A}-25 and CloneB^{6A} clones were the major ones (table 2). This could be the consequence of differences in spreading trends of clonal types producing AOM with respect to those producing invasive infections.

MLST was performed on clones which showed higher frequencies and on those that were associated with some international clones. All the isolates belonging to the Sweden^{15A}-25 clone were ST782 (SLV63) and clustered to CC63. CloneB^{6A} was ST473 and clone England^{14}-9 was ST9. Clone Spain^{9V}-3 was ST162, a single locus variant of ST156. Clone Poland^{6B}-20 was ST315 (table 2). Although PFGE patterns were identical to the Sweden^{15A}-25 and Spain^{9V}-3 clones, when we performed MLST, we noted that our isolates had different ST but belonged to highly related clonal complexes.

Sweden^{15A}-25 (CC63) is a worldwide disseminated clone usually showing capsular switching (serotype 19F or 19A). This clone has been described in Spain as a cause of meningitis and among multidrug- and ciprofloxacin-resistant strains with different serotypes (14, 19A, 19F, and 23F). In previous European studies[4], the Sweden^{15A}-25 clone was the most frequently found clone among erythromycin-resistant pneumococci (www.mlst.net). The MLST database includes several ST63 isolates recovered from AOM in France. However, this database does not contain ST782 isolates from AOM. In the present study, the Sweden^{15A}-25 clone was also the most frequently found clone expressing serotype 14, probably due to a previous capsular switching event.

Alonso *et al*. studied pneumococcal isolates causing AOM in a region of Northern Spain between 1999 and 2010. The authors detected that clones ST63 and ST156 were only occasionally present in their region, the last one associated with ERY-susceptible isolates[1]. Bowers *et al*. reported the dominance of multidrug-resistant CC271 clones in macrolide-resistant pneumococci in Arizona, principally due to the dual genotype (*mef*E/*erm*B)[3]. Among the *mef* positive isolates, they found two belonging to the ST156 clone, one of them showing serotype 9V, similar to clone D isolates found in our study, and one isolate belonging to the ST162 clone. Among the ermB positive isolates, they found eight ST63 and two ST315, with serotype 6B, similar to clone I.

This study was carried out before the introduction of the PCV13 vaccine in our immunization schedule in 2012; therefore, it deserves to be repeated later, to evaluate the vaccine effectiveness and the possible spread of new *S. pneumoniae* clones in AOM.

Although macrolides or lincosamides are not first-line antibiotics for the treatment of AOM, the knowledge about the prevalence of different macrolide-resistant mechanisms allowed us to assess and eventually predict their dissemination among pneumococci.

Although in our country we have data on the evolution of macrolide-resistant *S. pneumoniae* since 1995 from invasive infections and AOM [6], this is the first study assessing the mechanisms of macrolide resistance and genetic relatedness of *S. pneumoniae* isolated from pediatric patients suffering from AOM in Argentina.

Ethical approval

This research was approved by the Ethics Committee and the Dirección Asociada de Docencia e Investigación (Teaching and Research Associated Board) at the Hospital de Pediatría "Prof. Dr. Juan P. Garrahan".

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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