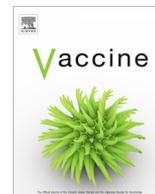




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Characterization of *Streptococcus pneumoniae* invasive serotype 19A isolates from Argentina (1993–2014)

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ABSTRACT

The aim of this study was to characterize *Streptococcus pneumoniae* serotype 19A isolates causing invasive pneumococcal disease in children, collected in Argentina between 1993 and 2014. A total of 176 isolates serotype 19A were analyzed. There was an increase in the proportion of serotype 19A isolates from 3% in 1993 to 6% in 2011, prior to the introduction of PCV13 in 2012, and from 2012 to 2014 its proportion gradually decreased.

Penicillin resistance among serotype 19A isolates throughout the study period was 65.9%, but a significant increase was observed from 0% in 1993 to 87.5% in 2014. Genetic relationship of the isolates was determined by PFGE and selected strains were studied by MLST. Most of the isolates belonged to two clonal types: A (54.5%) and B (11.4%). Isolates of clonal type A were ST1131, a single locus variant of ST172 and accounted for 54% of the total collection. They were detected for the first time in our country in 1997 and most of them (93%) were penicillin non susceptible. Isolates of clonal type B were ST8121, a single locus variant of ST199, and were mainly susceptible to penicillin. These two clonal types are still in circulation and appear to be responsible for the dissemination of *S. pneumoniae* serotype 19A invasive isolates in our country.

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1. Introduction

Streptococcus pneumoniae is an important bacterial pathogen to cause invasive pneumococcal diseases (IPD) in children, including bacteremia and meningitis. It has been estimated that pneumococcal disease causes approximately 1.6 million deaths each year and about half of these are children aged less than five years old [1,2]. To reduce the burden of pneumococcal disease, different formulations of pneumococcal conjugate vaccines have been introduced in many countries. In the year 2000 the 7-valent pneumococcal conjugate vaccine (PCV7) against serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F was licensed in the United States for young children. Their introduction in some countries decreased substantially the incidence of invasive pneumococcal disease caused by vaccine serotypes [3]. In Argentina, a 13-valent formulation (PCV13) that

includes serotypes 1, 3, 5, 6A, 7F, and 19A in addition to the serotypes contained in PCV7, was included in the National Immunization Program in January 2012 for children less than 2 years of age in a 2 + 1 schedule and catch-up. Despite the availability of these vaccines, pneumococcal infections remain a global problem due to the replacement of vaccine by non-vaccine serotypes, mostly associated with the emergence of multidrug resistant serotypes, such as the serotype 19A [4]. In the last few years some reports have shown that serotype 19A is becoming an important cause of pneumococcal disease in the United States population with high PCV7 vaccine uptake [5]. However, recent reports documented the same trend in geographic areas where PCV7 was not available, suggesting that other nonvaccine factors may play a role in this increase [6]. Genotypic characterization of serotype 19A isolates by multilocus sequence typing (MLST) showed that there are five major clonal complexes (CCs) associated with them, CC81, CC193, CC199, CC276, and CC320. Additionally, some of these clones are multidrug resistant, causing great concern [7]. Since 1993, the National IPD Surveillance Program has been conducted in Argentina in children less than 6 years old, as part of SIREVA II-PAHO network [8]. The main objectives of this network are to

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identify the prevalence of *S. pneumoniae* serotypes, to determine their antibiotic resistance profile and to evaluate the spread of high risk clones. As a result, serotype 19A accounted for 3% of invasive pneumococci received at the National Reference Laboratory until 2005. After that, its proportion rose progressively to 6–7%. The aim of this study was to characterize the invasive *S. pneumoniae* serotype 19A isolates collected from children in Argentina between 1993 and 2014.

2. Methodology

2.1. Population studied

From 1993 to 2014, the National Reference Laboratory (NRL) received 4391 invasive pneumococci from 101 hospitals in 20 provinces and Buenos Aires city from Argentina, 176 of them were serotype 19A. Samples were recovered from children under six years old from normally sterile sources and only one isolate per patient was included in this study. Isolates were collected from patients with pneumonia (49%), meningitis (19%), sepsis (14%), and other diagnosis (18%). Informed consent was not requested because it was a surveillance study.

2.2. Serotyping

Serotyping was based on the Neufeld Quellung reaction using antisera produced by the Statens Serum Institute (Copenhagen, Denmark).

2.3. Antimicrobial susceptibility testing

Susceptibility testing was determined by agar dilution method to penicillin, amoxicillin, cefotaxime, meropenem, ceftaroline (only for isolates from 2014), erythromycin, tetracycline, doxycycline (only for isolates from 2013 and 2014), chloramphenicol, trimethoprim-sulfamethoxazole, levofloxacin, rifampin and vancomycin, according to the CLSI guidelines [9,10]. *S. pneumoniae* ATCC 49619 was used as a quality control strain. Double disk diffusion assay using erythromycin (15 µg) and clindamycin (2 µg) disks was performed to evaluate the inducible or constitutive expression of the MLS_B phenotype [11]. Isolates with intermediate or high level resistance were defined as nonsusceptible and multidrug resistant (MDR) was defined as having intermediate or full resistance to three or more different classes of antibiotics. External quality assurance was done by the Adolfo Lutz Institute (Sao Paulo, Brazil).

2.4. Periods of study

In Argentina, PCV13 vaccination was introduced in January 2012 in a two-dose scheme (2 and 4 months) plus a booster at 12 months. The catch-up campaign at time of introduction was 2 doses for ages 12–24 months. In this context, we considered pre-vaccination period from 1993 to 2011 and post-vaccination period from 2012 to 2014.

2.5. Statistical analysis

Data were analyzed using EpiInfo and WHONET 5.4 (WHO). Statistically significant associations between population type and antimicrobial resistance were determined using chi-square or Fisher exact test. *p* value <0.05 was considered statistically significant.

2.6. Molecular characterization

Presence of macrolide/lincosamides resistance genes *ermB*, *mefA* and *lnuB* were identified by PCR as previously described [12]. Genetic relationship of all serotype 19A isolates was performed by *Sma*I-PFGE as previously described method [12] and interpreted using the Tenover criteria [13]. PFGE patterns were compared with those of representative international pneumococcal clones (clones 1–20 and 26) of the Pneumococcal Molecular Epidemiology Network [14]. A subset of strains from representative patterns defined by PFGE was studied by MLST as described previously [15]. Allele number and sequence types (STs) were assigned using the pneumococcal MLST web site [15].

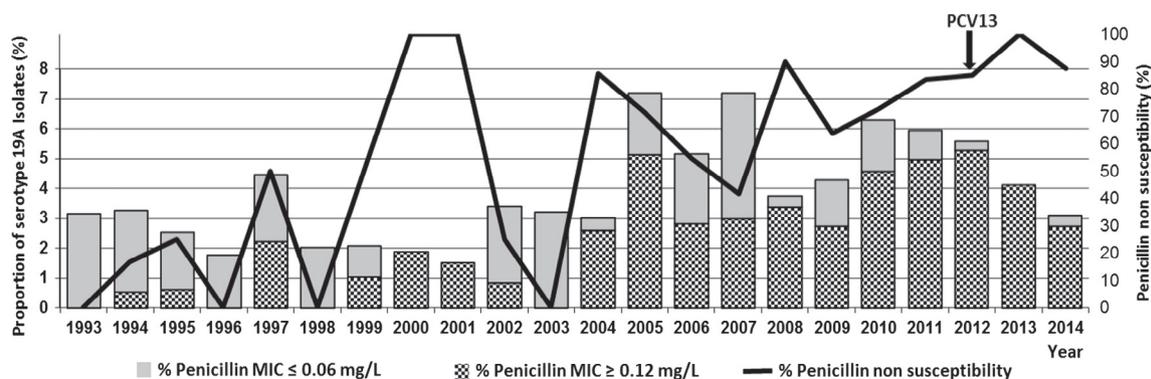
3. Results

3.1. Isolates

S. pneumoniae serotype 19A isolates from IPD increased from 3% in 1993 to 6% in 2011 ($p < 0.001$), in the pre-vaccination period. In the post-vaccination period, the proportion of serotype 19A isolates gradually decreased to 3% ($p < 0.001$) (Fig. 1). The average was 4% considering the entire study period.

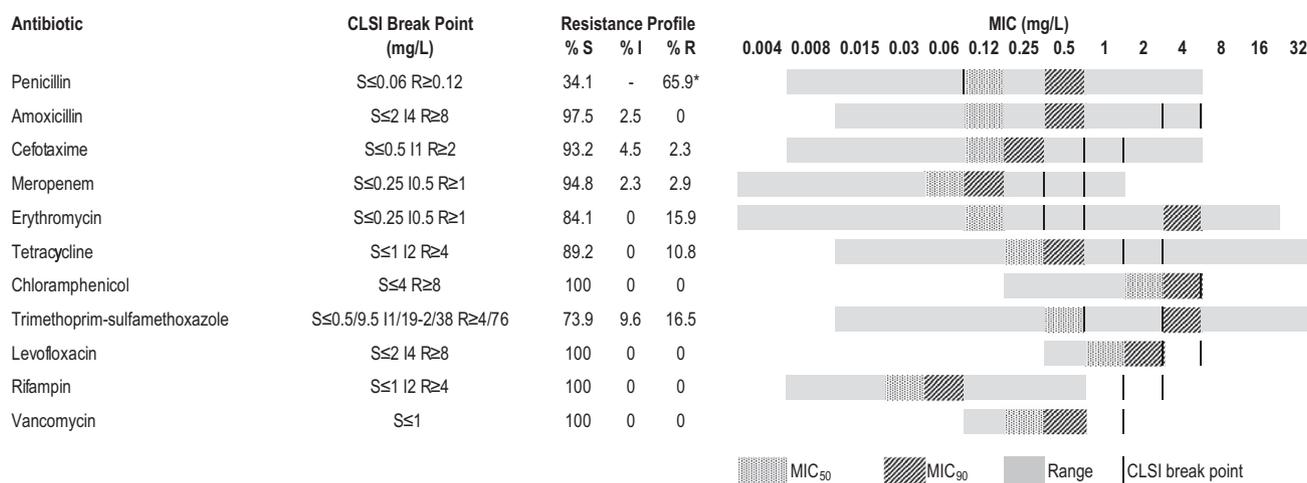
3.2. Antimicrobial resistance

MIC50, MIC90, range and resistance profile of the 176 *S. pneumoniae* 19A isolates are presented in Fig. 2. Considering the meningitis penicillin breakpoints, a total of 116 *S. pneumoniae* serotype 19A isolates (65.9%) were resistant to penicillin (MIC ≥ 0.12 mg/L).



Introduction of PCV-13 is indicated in the figure

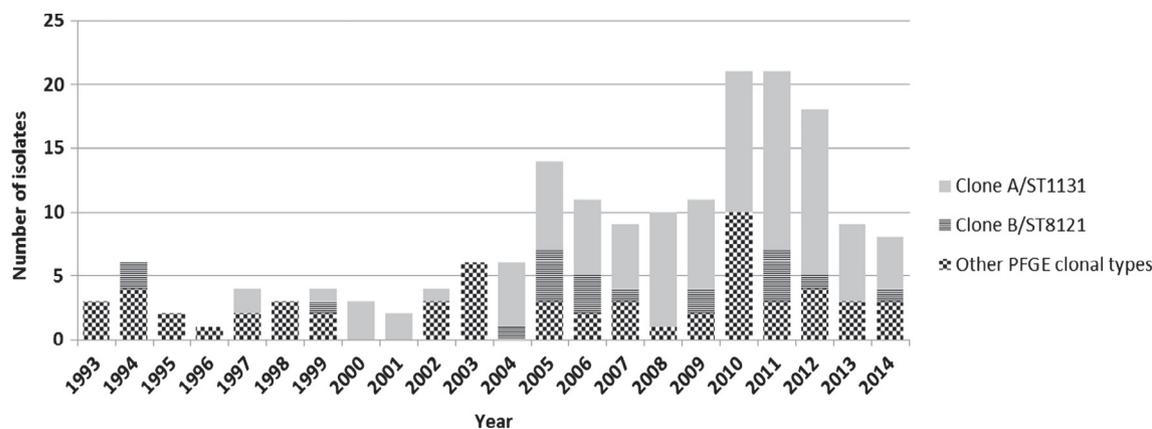
Fig. 1. Proportion of *S. pneumoniae* 19A isolates by year and penicillin non-susceptibility.



S:susceptible;I: intermediate;R resistant

The gray shaded bars indicate the range for each antibiotic, including MIC₅₀ (dotted bars), MIC₉₀ (hashed bars) and CLSI break point (black lines).

Fig. 2. MIC₅₀, MIC₉₀, range and resistance profile of 176 *S. pneumoniae* 19A isolates.



The bars indicate the number of isolates, in each clonal group.

Fig. 3. PFGE clonal types and MLST distribution of Argentinean invasive serotype 19A *S. pneumoniae* isolates throughout the period of study.

L), 105 (59.7%) with MIC between 0.12 and 1 mg/L and 11 (6.2%) with MIC \geq 2 mg/L. Penicillin resistance increased from 0% in 1993 to 87.5% in 2014 ($p < 0.001$) (Fig. 1).

Considering the meningitis breakpoints, 164 (93.2%) were susceptible to cefotaxime, 8 (4.5%) were intermediate and 4 (2.3%) were resistant. While, if applying the nonmeningitis breakpoints 172 (97.7%) were susceptible, 3 (1.7%) were intermediate and 1 (0.6%) was resistant to cefotaxime. All the isolates were susceptible to rifampin, levofloxacin, chloramphenicol and vancomycin. Cefazolin was tested from 2014 and all tested isolates were susceptible. Doxycycline was tested from 2013 and all tested isolates showed the same susceptibility profile as tetracycline. MDR was detected in 9.1% of the strains. The most common MDR phenotype was resistance to penicillin, cefotaxime, erythromycin, trimethoprim-sulfamethoxazole and tetracycline, detected in 6.3% of the isolates. Of the 28 erythromycin resistant strains, 15 (53.6%) presented the M phenotype, displaying erythromycin resistance and carried the *mefA* gene. The remaining 13 isolates (46.4%) presented the constitutive MLS_B phenotype (cMLS_B), displaying

simultaneous resistance to erythromycin and clindamycin, 5 of them carried the *ermB* gene and 8 contained the dual mechanism *ermB* plus *mefA* genes. *lnuB* gene was not detected.

3.3. Molecular characterization

By PFGE the 176 *S. pneumoniae* serotype 19A isolates were discriminated in 45 clonal types. Fig. 3 shows distribution of the isolates from dominant PFGE/MLST clonal types throughout the period of study. Most of the isolates (116; 65.9%) belonged to two clonal types: A (96; 54.5%) and B (20; 11.4%). Isolates of PFGE type A were assigned to ST1131, a single locus variant of ST172. They were detected for the first time in 1997 and stayed during the all period of study. Most of the ST1131 isolates (93%) were penicillin non susceptible and presented penicillin MICs between 0.12 and 0.5 mg/L. Isolates of ST1131 presented in their majority susceptibility to all the antibiotics tested except penicillin (Table 1).

Table 1
Relationship between sequence type / PFGE clonal type and antimicrobial resistance of 176 serotype 19A *S. pneumoniae* isolates.

PFGE clonal type/ST	n	PEN-R		CTX-R		ERY-R		TET-R		SXT-NS	
		n	%	n	%	n	%	n	%	n	%
Clonal type A/ST 1131	96	92	95.8	0	0	0	0	1	1	8	8.3
Clonal type B/ST 8121	20	2	10	1	5	5	25	0	0	8	40
Other 43 minor PFGE clonal types	60	22	36.7	11	18.3	23	38.3	18	30	30	50

ST: sequence type; n: number of isolates; R: resistant; NS: non susceptible; PEN: penicillin; CTX: cefotaxime; ERY: erythromycin; TET: tetracycline; SXT: trimethoprim-sulfamethoxazole.

Isolates of PFGE type B were assigned to ST8121, a single locus variant of ST199. These isolates represent 11.4% of the total collection and most of them (90%) were susceptible to penicillin, 40% were resistant to trimethoprim-sulfamethoxazole and 25% to erythromycin (Table 1). Isolates resistant to macrolides were highly diverse, 23 of them (82%) were related to 20 PFGE clonal types other than ST1131 and ST8121.

4. Discussion

The prevalence of serotype 19A *S. pneumoniae* in Argentina during the study period was 4%. Our results were similar to those obtained in a meta-analysis performed by Castañeda *et al.* in 20 countries of Latin America and Caribbean between 1993 and 2009 where the global prevalence of this serotype was 3.8% [16].

Regardless of the fact that the introduction of PCV7 in the United States caused a decrease in both invasive and noninvasive pneumococcal diseases, increased rates of IPD with *S. pneumoniae* serotype 19A were reported [17,18]. There are many theories about the increase of serotype 19A isolates in the world, mainly associated to vaccine induced serotype replacement, antibiotic pressure, introduction of new clones and/or the increase of previously circulating clones [7]. However, serotype 19A increase due to the introduction of the PCV7 is controversial because it was also observed in countries without vaccination, such as southern Israel and South Korea [6]. Moreover, recent studies from Spain and Portugal, where the vaccine is available but is not part of the national vaccination plan, have shown that even relatively modest vaccination coverage rates had a profound effect on the serotypes responsible for invasive infections in children and adults [19].

PCV7 was available in Argentina from 2000, but its use has depended of the paediatrician recommendation and family decision. Nevertheless, an increase of infections caused by serotype 19A has been noted in the paediatric population. PCV13 has been introduced in the National vaccination schedule in January 2012 for children less than 1 year old using a 2 + 1 schedule and catch-up between 12 and 24 months. Despite the short post-vaccination period evaluated, our results showed a significant decrease of the serotype 19A (Fig. 1).

Recently, a study was carried out in Argentina evaluated the PCV13 impact during the two years following their introduction in the National Calendar of vaccination (2012–2013) compared to the baseline period 2003–2005 [20]. The authors found a significant reduction in the incidence of consolidated pneumonia (per 100,000 children < 5 years) between pre- and post-vaccination periods from 750 (204/27 209) to 561 (171/30 475) in 2012 and 453 (138/30 475) in 2013, with an effectiveness ratio of 25.2% and 39.6%, respectively. The reduction in children less than 1 year was 33.9% in 2012 and 44.6% in 2013; and 57.9% in children of 12–23 months in 2013.

The clonal complexes associated with serotype 19A infections worldwide are CC81, CC193, CC199, CC230 and CC320 [7,19]. The lineages driving the rise of serotype 19A infections in Asia and the United States are ST320 and ST199. The major clonal complex

found in the United States, CC320, was also a major lineage in South Korea [21], and the most frequent ST found in a recent study from Colombia [22]. In contrast, in Europe, CC230 has consistently been identified as a major serotype 19A lineage causing invasive infections before vaccine introduction [3]. In our country, the most prevalent clone among the invasive serotype 19A isolates was ST1131, a single-locus variant of the ST172 (Fig. 3). When compared to the reference MLST database only three serotype 19A isolates ST1131 were found, from Uruguay, Spain and Brazil. One strain, serotype 19F, isolated in Greece in 2006 has the same ST, probably as a result of capsular switching. In the present study, most of the ST1131 isolates (93%) were penicillin non susceptible and 96% of them presented penicillin MICs between 0.12 and 0.5 mg/L. Similar penicillin MICs values were exhibited by the ST1131 isolates found in the database. Additionally, ST1131 was the main serotype 19A clone associated with isolates causing acute otitis media infections and isolates carried by healthy children attending day care centers in Argentina between 2007 and 2008 (Corso A, personal communication). ST172 was previously detected in Israel, in a study performed with children with acute otitis media between 1999 and 2006. In that study, ST172 isolates represented 69.4% of all tested [6]. A more recent study performed on the same country showed that ST172 is also the dominant clone among serotype 19A IPD isolates [23]. A similar situation was observed in Alaska, in a study characterizing invasive isolates where serotype 19A ST172 was detected. This clone emerged in 2000 after introduction of PCV7 and represented 34% of 19A isolates from 2001 to 2010 [24]. In the United States ST172 was found in bacteraemic patients but in serotypes 19A and 23F [23,25]. Indeed, ST172 isolates within the pneumococcal MLST database has been associated with other serotypes, suggesting that capsular switching may occur.

The second most frequent ST detected in our study represented 11.4% of the isolates was ST8121 (Fig. 3), a single-locus variant of the ST199. At the moment, there are no other isolates with this ST loaded on the MLST database.

When *Sma*I-PFGE profiles of all isolates were compared with representative international pneumococcal clones, only one strain was related to Sweden^{15A}-25/ST782, a single locus variant of ST63. Previously we studied a collection of macrolide resistant *S. pneumoniae* isolated from pediatric patients with acute otitis media and we found that ST782 represented 27% of the isolates studied. They carried *mefA* genotype but were serotype 14 instead of 19A [26].

During the last years, the proportion of penicillin non-susceptible and MDR serotype 19A isolates increased in many countries [24,27]. In the United States CC199 is being replaced by CC320. The increase in the percentage of CC320 isolates relative to the percentage of CC199 isolates within serotype 19A is concordant with the increase of penicillin resistant rates observed in the last years [27]. Our results showed that the resistance to β -lactams in serotype 19A *S. pneumoniae* isolates from Argentina increased significantly over time. This could be explained by the increase in the proportion of ST1131 isolates which were uniformly intermediate-resistant to penicillin.

In our study, the macrolide resistant isolates represent 15.9% (28/176) and belong to 21 different clonal types. Most of macrolide resistant isolates (23/28, 82.1%) carried the *mefA* gene, but 8 of them were positive for both *ermB* and *mefA* with MLS_B phenotype. MDR is commonly observed in serotype 19A *S. pneumoniae* isolates. Studies performed in Asia, Spain and the United States, showed high MDR phenotype in *S. pneumoniae* serotype 19A isolates [28–30]. In this study, MDR was detected only in 9% of the serotype 19A isolates and it was found associated with minor clonal types (Table 1) and not with the major clones ST1131 and ST8121.

In summary, the increase of *S. pneumoniae* serotype 19A in our country was mainly due to the dissemination of ST1131 and ST8121. Although we cannot evaluate the effect of partial vaccination with PCV7 and the use of antibiotics, our findings suggest that the increase in serotype 19A was in part associated with the dissemination of preexisting clones circulating before the introduction of PCV13 into the national calendar of vaccination. Our data highlight the importance of continuous surveillance to assess the impact of pneumococcal vaccines and the use of antibiotics in the dynamics of pneumococcal clones. Further studies are needed within a few years to assess changes in the pneumococcal population following the introduction of PCV13 in Argentina.

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Conflict of interest

We have no conflict of interests to declare.

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