Emergence of a multidrug-resistant clone (ST320) among invasive serotype 19A pneumococci in Spain

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Objectives: Multidrug-resistant Streptococcus pneumoniae isolates of serotype 19A have emerged all over the world in recent years. The aim of this study was to characterize highly penicillin-resistant pneumococcal strains of the 19A serotype, collected in Spain from 1997 to 2007 from patients with invasive disease.

Methods: Antibiotic susceptibility was studied by microdilution. All penicillin-resistant pneumococci were typed by PFGE and selected strains were studied by multilocus sequence typing (MLST). The presence of genes related to the Tn916 family of transposons was investigated by PCR.

Results: From a total of 1197 invasive pneumococcal isolates of serotype 19A received at the Spanish Reference Laboratory between 1997 and 2007, 51 (4.3%) strains showed high-level resistance to penicillin (MICs of 2–4 mg/L). These 51 isolates belonged to three multiresistant clones related to sequence type (ST)81 (n = 21), ST320 (n = 19) and ST276 (n = 11). All 51 serotype 19A pneumococci were tetracycline-resistant and had the tet(M) gene, and 41 strains were macrolide-resistant, harbouring the erm(B) gene. The presence of int and xis genes was detected in all strains associated with other genes of the Tn916 family.

Conclusions: The rise in penicillin-resistant serotype 19A invasive pneumococci in Spain was associated with the emergence and clonal spread of two worldwide-disseminated multiresistant clones (ST276 and ST320). The Spain23F-1-19A (ST81) clone remained stable throughout the study period. Multidrug resistance was associated with transposons of the Tn916 family.

Keywords: Streptococcus pneumoniae, resistance, transposons

Introduction

Multidrug-resistant serotype 19A Streptococcus pneumoniae isolates have been recorded all over the world in recent years, especially in countries where seven-valent pneumococcal conjugate vaccine (PCV7) has been introduced for children.1–4 The presence of serotype 19A has also increased in countries without vaccine introduction, such as southern Israel and South Korea.5,6 In the USA, the increase in serotype 19A has been mainly associated with two clonal complexes (CCs), CC199 and CC320. Multiresistant CC320 emerged in the USA after PCV7 introduction, whereas CC199, which was already well established in that country in the late 1990s, expanded in the PCV7 period.1,4

In Spain, PCV7 was introduced in June 2001, and the current estimated PCV7 uptake of children under 2 years is ~50%.7 Until 2002, serotype 19A accounted for <5% of invasive pneumococci received at the Spanish Reference Laboratory, but between 2003 and 2007 its incidence rose progressively to 10%.7 Fifty-one of these strains were penicillin- (MICs of 2–4 mg/L) and multidrug-resistant. The aims of this study were: (i) to analyse the genotypes of these 51 penicillin-resistant
invasive pneumococci of serotype 19A isolated during an 11 year period; and (ii) to analyse the presence of transposons related to the Tn916 family in these multidrug-resistant strains.

Materials and methods

From 1997 to 2007, the Spanish Reference Laboratory for Pneumococci received 17112 invasive pneumococci from all over the country, of which 1197 were serotype 19A. In the present study, the 51 serotype 19A isolates showing resistance to penicillin (MIC of ≥2 mg/L) were analysed.

Antibiotic susceptibility testing

MICs were determined by the CLSI microdilution method with Mueller–Hinton broth containing 2%–5% lysed horse blood, following international recommendations and using commercially available panels (STRHELP; Sensititre, West Sussex, UK). S. pneumoniae ATCC 49619 was used for quality control. The following antibiotics (range of dilutions) were tested: penicillin (0.03–8 mg/L), cefotaxime (0.06–4 mg/L), erythromycin (0.25–32 mg/L), clindamycin (0.25–0.5 mg/L), trimethoprim/sulfamethoxazole (0.5/5–2/38 mg/L), chloramphenicol (2–8 mg/L) and tetracycline (2–4 mg/L).

Molecular typing

Genomic DNA was restricted with SmaI. Fragments were separated by PFGE in a CHEF-DRIII apparatus (Bio-Rad) as described previously. Molecular Epidemiology Network (http://www.sph.emory.edu/medinet/) was used for quality control. The following antibiotics (range of dilutions) were tested: penicillin (0.03–8 mg/L), cefotaxime (0.06–4 mg/L), erythromycin (0.25–32 mg/L), clindamycin (0.25–0.5 mg/L), trimethoprim/sulfamethoxazole (0.5/5–2/38 mg/L), chloramphenicol (2–8 mg/L) and tetracycline (2–4 mg/L).

Gene detection by PCR

Macrolide [erm(B), mef(A/E)] and tetracycline [tet(M)] resistance genes were studied by PCR applying methodology described elsewhere. The PCR products of the mef gene were digested with BamHI in order to discriminate between the mef(A) and mef(E) gene subclasses. This approach was unable to differentiate between mef(I) and mef(E) genes. To investigate the presence of the Tn916 family of transposons, int, xis, tnpA and tnpR genes and the promoter of the aph3–III gene were studied by PCR using conditions described previously.

Results and discussion

All 51 isolates showed resistance to ≥4 drugs (Figure 1). The MICs of penicillin and cefotaxime were in the range 2–4 and 0.5–4 mg/L, respectively. Resistance rates were as follows: tetracycline, 100%; co-trimoxazole, 78.4%; erythromycin, 80.4%; clindamycin, 80.4%; and chloramphenicol, 41.2%. These multidrug-resistant strains were classified in eight different PFGE patterns (Figure 1). Fourteen pneumococci were studied by MLST (at least one strain for each PFGE pattern). After MLST analysis three major clonal groups were identified: ST81, associated with three PFGE patterns (C-1, AB and AD) accounting for 21 isolates (5 from children and 16 from adults); ST320, associated with three PFGE patterns (P, AF and AG) accounting for 19 isolates (12 from children and 7 from adults); and ST276, associated with two PFGE patterns (Z and AC) accounting for 11 pneumococci (3 from children and 8 from adults).

During the study period the overall rate of penicillin resistance (MIC of ≥2 mg/L) decreased from 11.4% in 1997 to 10.3% in 1998, 9.1% in 1999, 10.1% in 2000, 9.1% in 2001, 7.8% in 2002, 5.9% in 2003, 8.1% in 2004, 6.7% in 2005, 4.2% in 2006 and 6.4% in 2007 (P<0.01). This decline in penicillin resistance rates has been associated with a decrease in multidrug-resistant PCV7 serotypes after the introduction of PCV7 for children in Spain. Figure 2 shows the contribution to penicillin resistance of the three major clones throughout the study period. The proportion of serotype 19A among penicillin-resistant strains progressively increased from 2.0% in 1997 to 12.1% in 2007 (P=0.03). This increase was especially noticeable in the 2005–2007 period (Figure 2).

Figure 1 shows the transposon-related genes detected by PCR, and the resistance patterns of serotype 19A pneumococci and genotypes. Among 21 isolates related to ST81 (genotypes C-1, AB and AD), the presence of Tn916 was presumed in 10 [tet(M)(+), int(+) and xis(+)], Tn6002 in 6 [erm(B)(+), tet(M)(+), int(+) and xis(+)] and Tn3872 in 5 [erm(B)(+), tet(M)(+), int(+), xis(+) and tnpR(+)]. Two isolates, one related to Tn6002 and the other to Tn3872, also harboured the mef(E) gene. As in previous reports, all but one of the serotype 19A isolates related to ST320 (genotypes P, AF and AG) had tet(M), int and xis genes and dual macrolide resistance [erm(B) and mef(E)], probably due to the presence of the recently described Tn2010. This transposon has been associated with CC271 pneumococci, including ST320. These pneumococci were isolated in the USA, South Korea, China and Brazil in the 1999–2000 period. Finally, among 11 strains whose genotypes were related to ST276 (genotypes Z and AC), 7 had a gene profile related to Tn6002 [erm(B)(+), tet(M)(+), int(+) and xis(+)] and 4 a gene profile related to Tn1545 [erm(B)(+), tet(M)(+), int(+), xis(+) and aph3(III)+], as described among macrolide-resistant pneumococci obtained in 2004 in Spain.

The most frequent genotype among 51 penicillin-resistant serotype 19A pneumococci was the Spain23F-1 clone, the result of capsular switching between serotypes 19A and 23F, named Spain23F-1-19A according to PMEN recommendations (http://www.sph.emory.edu/PMEN/index.html). Recent studies show a decline in the global rate of the Spain23F-1 (ST81) clone, associated with the dramatic fall of the 23F serotype after introduction of PCV7 in Spain. However, the rate of the Spain23F-1-19A clone remained stable among penicillin-resistant strains throughout this period, from 2.0% in 1997 to 1.2% in 2007 (P=0.58) (Figure 2).

Our results demonstrate that the pneumococci of serotype 19A related to ST320 showing high levels of resistance to penicillin and macrolides was first detected in Spain in 2005, mainly associated with invasive disease in children. This emerging clone increased significantly, accounting for 8.6% of penicillin-resistant invasive pneumococci recovered in 2007 (P=0.002), and now ranks first among penicillin-resistant serotype 19A pneumococci isolated in Spain (Figure 2). A recent South Korean study reports the presence of ST320 pneumococci expressing serotypes 19A and 19F in a Seoul hospital since the
The increase in serotype 19A among children <5 years old was associated with the spread of the ST320 clone before (1998–2003) and after (2004–2006) PCV7 introduction in South Korea. ST320 is a double-locus variant of the worldwide-established Taiwan 19F-14 (ST236) clone. This international clone, predominant among invasive pneumococci in Asian countries, was associated with the increase in penicillin and macrolide resistance rates in the 1990s in this region. In addition, CC320 pneumococci were associated with the increase in multidrug-resistant invasive serotype 19A pneumococci isolated in the USA after PCV7 introduction.

The third most frequent penicillin-resistant serotype 19A clone in our country was related to ST276, a single-locus variant of the ST230 identifier of the Denmark14-32 clone, which emerged in Spain in 2004. The prevalence of this clone among invasive pneumococci with a penicillin MIC of ≥2 mg/L increased from 0% in 1997 to 2.3% in 2007 but it did not reach statistical significance ($P = 0.12$). ST276 pneumococci have been previously described in Europe and the USA2,3,5 as well as among erythromycin-resistant serotype 19A strains collected in a nationwide study carried out in Spain.11 Capsular switching of the Denmark (ST230) clone with serotypes 19F, 19A and 24F has been reported in Spain and other countries.1,2,5,11 The expansion of pneumococci related to ST276 was associated with the increase in serotype 19A infection in French children after PCV7 introduction.2 In addition, the ST276 clone has recently been associated with an increase in

![Figure 1](https://academic.oup.com/jac/article-abstract/64/3/507/772679)

**Figure 1.** Dendrogram of the PFGE (SmaI) profiles using the Dice coefficient and UPGMA (Fingerprinting software). Each profile is associated with a PFGE pattern, a multiresistance pattern and transposon-related genes detected by PCR. PEN, penicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol; SXT, co-trimoxazole.

<table>
<thead>
<tr>
<th>No.</th>
<th>PFGE Pattern</th>
<th>Multiresistance Pattern</th>
<th>Gene Detection by PCR</th>
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<td>ATCC 700669</td>
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![Figure 2](https://academic.oup.com/jac/article-abstract/64/3/507/772679)

**Figure 2.** Distribution of penicillin-resistant serotype 19A isolates belonging to different clones throughout the study period. The bars indicate the proportion of each serotype 19A clone among penicillin-resistant pneumococci. PenR, penicillin-resistant; ST, sequence type.

The increase in serotype 19A among children <5 years old was associated with the spread of the ST320 clone before (1998–2003) and after (2004–2006) PCV7 introduction in South Korea. ST320 is a double-locus variant of the worldwide-established Taiwan 19F-14 (ST236) clone. This international clone, predominant among invasive pneumococci in Asian countries, was associated with the increase in penicillin and macrolide resistance rates in the 1990s in this region. In addition, CC320 pneumococci were associated with the increase in multidrug-resistant invasive serotype 19A pneumococci isolated in the USA after PCV7 introduction.

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otitis media infections due to serotype 19A in Bedouin children in southern Israel, a population without PCV7 immunization. In conclusion, the rise in highly penicillin-resistant serotype 19A pneumococci observed in Spain in 2007 was associated with the emergence and clonal spread of two worldwide-disseminated multiresistant clones related to ST276 and ST320, filling the gap left by the multidrug-resistant PCV7-related clones. In contrast, the Spain 23F-1-19A (ST81) clone remained stable throughout the study period. Further molecular surveillance studies of invasive pneumococci are needed to assess the impact of pneumococcal vaccines and antibiotic use on the dynamics of pneumococcal clones.

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Transparency declarations

None to declare.

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