Antimicrobial susceptibility and pneumococcal serotypes

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The increase in antibiotic resistance and the possible changes in serotype prevalence as a consequence of a new conjugated vaccine have contributed to renewed interest in the study of pneumococcal serotypes and their antibiotic resistances. Spain still has one of the highest penicillin resistance rates, but in the past 4–5 years a slight decrease has been observed. The level of resistance has not increased either, 12.7% of the 11 165 isolates studied showed high-level penicillin resistance but 94% of these had an MIC of only 2 mg/L. Serotypes 6, 9, 14, 19 and 23 included 83% of the penicillin-resistant pneumococci; the remaining 17% belonged to 18 different serotypes. We analysed these minor penicillin-resistant serotypes in view of their potential increase following a possible child vaccination programme. Four of these serotypes (11, 15, 21 and 35) were the most prevalent, and among them serotype 15 was particularly frequent with >50% of its strains resistant. The effective control of these minor penicillin-resistant serotypes should be based on continuous surveillance of pneumococcal epidemiology.

Introduction

Streptococcus pneumoniae is an important cause of concern because of the high incidence of pneumococcal infections and the widespread increase in antibiotic-resistant strains.1-5 The continuous surveillance of pneumococcal serotypes and of their resistance to antibiotics is now common practice in many countries as it provides the necessary information for effective control of these infections. Moreover, the recent marketing of a seven-valent conjugate vaccine for use in children under 2 years of age2 illustrates the renewed interest in pneumococcal serotype studies. A reduction in carriage of vaccine serotypes and, as they are the most frequent penicillin-resistant serotypes, a reduction in the percentage of penicillin-resistant pneumococci, have also been observed after vaccination.7,8 Although there is some evidence that vaccine types are replaced in the pharynx of carriers by non-vaccine serotypes, the effects of widespread vaccination on the epidemiology (serotypes and resistance) of pneumococcal populations are still uncertain.9 The purpose of this study was to analyse the current situation in Spain with respect to the prevalence of serotypes causing disease and to antibiotic resistance. Attention is focused on the evolution of penicillin-resistant serotypes.

Materials and methods

Pneumococcal strains received between January 1997 and July 2001 at the reference laboratory were included in the study. Only one isolate per patient was included. Strains were confirmed as S. pneumoniae by standard methods and serotyping was performed by Quellung reaction and/or dot blot assay,10 using antisera from Statens Seruminstitut, Copenhagen, Denmark.

Susceptibilities to antibiotics were determined by the agar dilution technique as described previously,11 and interpreted according to criteria from the National Committee for Clinical Laboratory Standards (NCCLS).12 Unless otherwise specified, the term penicillin-resistant pneumococci (PRP) refers to both moderately and fully resistant strains.

Results

Between January 1997 and June 2001, 11 165 pneumococcal strains isolated in 113 Spanish hospitals throughout the country were received for serotyping and antibiotic susceptibility surveillance. Laboratories sent 100% of their isolated strains. These strains were sent on a voluntary basis so regions were represented differently.11 There were no data on patient age

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for 566 strains; 7745 strains were isolated from adults and 2854 from children. The sources of the pneumococci are shown in Figure 1. The majority of the pneumococci isolated from blood, sputum and lower respiratory tract (pleural fluid, bronchial aspirate and bronchoalveolar lavage) were from adult patients, while those from ear, nose, eye and pharynx samples were isolated from children.

Overall, 40 different serotypes were identified, although 90% of the strains belonged to 20 serotypes and only six of them accounted for 60.4% of all pneumococci. The 10 most common serotypes, in decreasing order, were: 19 (12.8%), 6 (11.7%), 3 (10.1%), 23 (9.7%), 14 (9.4%), 9 (6.7%), 4 (3.7%), 1 (3.6%), 15 (3.4%) and 18 (3.1%). Ninety-four per cent of the serotypes identified were included in the 23-valent pneumococcal polysaccharide vaccine, of which only 60% are included in the current seven-valent conjugate vaccine. When pneumococci isolated only from children were considered, this coverage rose to 75.6%.

Table 1 shows the in vitro susceptibility of pneumococcal isolates. Using the current breakpoints established by the NCCLS,12 47.5% of the strains were resistant to penicillin, 7.8% to amoxicillin, 21.3% to cefotaxime, 38.3% to tetracycline, 22.1% to chloramphenicol and 34.8% to erythromycin. Twenty-three of 1500 (1.5%) pneumococci showed resistance to levofloxacin. Nevertheless, among the antibiotics tested, cefotaxime and levofloxacin had the lower MIC90s. The annual percentage of pneumococci with intermediate and high resistance to penicillin and cefotaxime is shown in Table 2. PRP decreased from 53% in 1997 to 43.9% in the first half of 2001, but no changes were observed in the incidence of pneumococcal resistance to cefotaxime. The decrease in percentage of PRP occurred in the intermediate resistance category (MIC 0.12–1 mg/L), while the percentage of strains with penicillin MICs of >1 mg/L remained unchanged.

Prevalence of PRP by serotype

Among the 5310 PRP detected during the period studied, 83% belonged to five serotypes: 6 (18%), 9 (12%), 14 (19%), 19 (19%) and 23 (15%), which are classically associated with penicillin resistance, and the remaining 17% were distributed in 18 different serotypes. Table 3 illustrates the serotypes of the PRP found in our laboratory since 1979. PRP have been grouped into two categories: strains belonging to penicillin resistance-associated serotypes (PRA-STs) and those belonging to other STs. Apart from the five PRA-STs, penicillin-resistant strains have been detected in a total of 24 different serotypes at any time. Some serotypes appeared sporadically, but others were consistently recovered in all or nearly all periods. Except for serotypes 11, 15, 21 and 35, plus non-

### Table 1. *In vitro* activity of antibiotics against 11165 pneumococcal isolates

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC range (mg/L)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (mg/L)</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt; (mg/L)</th>
<th>No. (%) of resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>0.015–8</td>
<td>0.06</td>
<td>2</td>
<td>5310 (47.5)</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>≤0.06–16</td>
<td>≤0.06</td>
<td>2</td>
<td>261 (7.8)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.015–16</td>
<td>0.06</td>
<td>1</td>
<td>2381 (21.3)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.12–128</td>
<td>0.5</td>
<td>64</td>
<td>4281 (38.3)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1–32</td>
<td>4</td>
<td>16</td>
<td>2462 (22.1)</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.06–≥128</td>
<td>0.12</td>
<td>≥128</td>
<td>3888 (34.8)</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>≤0.5–≥32</td>
<td>1</td>
<td>1</td>
<td>23 (1.5)</td>
</tr>
</tbody>
</table>

*Breakpoints as defined by the NCCLS.12

13348 isolates were tested.

1500 isolates were tested.
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Table 2. Penicillin and cefotaxime non-susceptible pneumococci (1997–2001)

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of strains</th>
<th>No. (%) of strains with penicillin MIC (mg/L)</th>
<th>No. (%) of strains with cefotaxime MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.12–1</td>
<td>≥2</td>
</tr>
<tr>
<td>1997</td>
<td>1812</td>
<td>720 (39.7)</td>
<td>261 (13.3)</td>
</tr>
<tr>
<td>1998</td>
<td>1917</td>
<td>720 (37.6)</td>
<td>261 (13.3)</td>
</tr>
<tr>
<td>1999</td>
<td>2737</td>
<td>953 (34.8)</td>
<td>315 (11.5)</td>
</tr>
<tr>
<td>2000</td>
<td>2725</td>
<td>889 (32.6)</td>
<td>343 (12.6)</td>
</tr>
<tr>
<td>2001†</td>
<td>1974</td>
<td>612 (31.0)</td>
<td>255 (12.9)</td>
</tr>
<tr>
<td>1997–2001</td>
<td>11165</td>
<td>3894 (34.8)</td>
<td>1416 (12.7)</td>
</tr>
</tbody>
</table>

†January to June.

Table 3. Serotypes of penicillin-resistant pneumococci (MICs > 0.06 mg/L)

<table>
<thead>
<tr>
<th>Periods (year)</th>
<th>Total no. (%)</th>
<th>No. (%) of PRA-STs</th>
<th>No. (%) of pneumococci belonging to other STs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1979–1981 n = 430</td>
<td>83 (19.3)</td>
<td>50 (60.2)</td>
<td>33 (39.8)</td>
</tr>
<tr>
<td>1982–1984 n = 951</td>
<td>323 (34.0)</td>
<td>271 (83.9)</td>
<td>52 (16.1)</td>
</tr>
<tr>
<td>1985–1987 n = 1191</td>
<td>458 (38.5)</td>
<td>399 (87.1)</td>
<td>59 (12.9)</td>
</tr>
<tr>
<td>1988–1990 n = 2165</td>
<td>1031 (47.6)</td>
<td>898 (87.1)</td>
<td>133 (12.9)</td>
</tr>
<tr>
<td>1991–1993 n = 4133</td>
<td>1976 (47.8)</td>
<td>1728 (87.4)</td>
<td>248 (12.6)</td>
</tr>
<tr>
<td>1994–1996 n = 4331</td>
<td>2184 (50.4)</td>
<td>1845 (84.5)</td>
<td>339 (15.5)</td>
</tr>
<tr>
<td>1997–1999 n = 6466</td>
<td>3211 (49.7)</td>
<td>2652 (82.6)</td>
<td>559 (17.4)</td>
</tr>
<tr>
<td>2000–2001 n = 4699</td>
<td>2099 (44.7)</td>
<td>1763 (84.0)</td>
<td>336 (16.0)</td>
</tr>
</tbody>
</table>

PRA-STs, penicillin resistance-associated serotypes (6, 9, 14, 19 and 23).

Serotypes (STs) are listed in numerical order.

NT, non typeable strains.

AT, atypical strains: optochin-resistant and/or bile-insoluble isolates.

typeable (NT) and atypical (AT) strains, the number of isolates of each serotype in every period was <10, most of which were represented by only one or two strains. Moreover, the great majority of these pneumococci showed a low level of resistance (MIC < 0.5 mg/L).

Evolution of PRA-STs

Figure 2 represents the evolution of PRA-STs from 1979 to 2001 distributed in 3 year periods. Figure 2(a) shows the relative frequency of these strains in relation to the total pneumococci and Figure 2(b) the percentages of PRP within each PRA-ST. All five PRA-STs increased their frequency in the 1980s, but serotype 23 showed the most pronounced increase (from 5.6% in 1979–1981 to 18.4% in the period 1982–1984). Only serotype 19 pneumococci have steadily increased over time, representing the most frequent serotype (67% of serotype 19 became resistant (Figure 2b).

The 1990s almost all serotype 6 isolates remained resistant, but PRP within serotype 23 decreased. Serotypes 9, 14 and 19 acquired resistance to penicillin during the 1980s and, in the period 2000–2001, 81% of serotype 9, 95% of serotype 14 and 67% of serotype 19 became resistant (Figure 2b).

Figure 3 shows the evolution of serotypes 11, 15, 21 and 35, which were the most frequent penicillin-resistant serotypes apart from PRA-STs. Each of these four serotypes represented <4% of the isolates received every year. Serotype 15 was included among the 11 most common serotypes since 1981 and >50% of the strains have always been resistant. Serotypes 11 and 21 decreased in frequency over time, accounting for 2% and 0.3%, respectively, of the pneumococci studied in the later period. In the 1980s, 50% of serotype 11 and 83% of serotype 21 were penicillin resistant, but in subsequent years these percentages decreased. Rarely isolated until 1996, serotype 35 has become more frequent over recent years, and the proportion of PRP within this serotype has also increased. During the surveillance period, 1416 isolates showed a high level of resistance to penicillin; the serotypes of these strains are presented in Table 4. The great
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majority had an MIC of 2 mg/L and belonged to the five PRA-STs (94%). Seventy-five isolates had an MIC of 4 mg/L and only eight of 11 165 isolates had an MIC of 8 mg/L, and they were identified as atypical strains (non-capsulated, optochin resistant and/or bile insoluble that gave positive hybridization with autolysin and pneumolysin probes).

**PRP and forms of disease**

The contribution of PRA-STs to invasive and non-invasive disease in both children and adults and the proportion of them among PRP are summarized in Table 5. As can be seen, 62% of the pneumococci isolated from blood and CSF from children belonged to the five PRA-STs, but only 39% of invasive strains from adult patients were included in the PRA-ST group. The sharp differences in penicillin resistance observed between children and adults suffering invasive disease correlated well with the different proportion of PRA-STs in each age group. Among the PRP causing invasive disease in children and adults, nearly all, 96% and 92%, respectively, belonged to PRA-STs. To analyse non-invasive diseases, ear isolates from children and strains recovered from sputum in adult patients were compared (Table 5). Again, PRA-STs were more common among paediatric than among adult pneumococci. As many as 80% of all pneumococci causing acute otitis media in children belonged to PRA-STs and they were responsible for the very high occurrence of PRP in this group. In the adult population, the percentage of PRP was higher among pneumococci isolated from sputum (55.5%) than those from blood and CSF (33%). Twenty-two per cent of PRP isolated from sputum belonged to serotypes other than PRA-STs.

**Discussion**

The concern about the increase in antibiotic resistance and the marketing of a seven-valent conjugate vaccine have probably been the main reasons for the high increase in the number of hospitals sending pneumococcal isolates to the reference laboratory as part of the most recent surveillance study (62 hospitals in 1990–1996 compared with 113 in the present study). In general, serotypes causing disease during the current surveillance period were the same as those reported in
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previous studies\textsuperscript{11,13}, although some slight differences have occurred: serotypes 5 and 8 have decreased in frequency with respect to previous years; in contrast, serotype 19 is growing in number and represents the most frequent serotype isolated in Spain today.

Although Spain still has one of the highest rates of PRP, a stable situation was reached at the beginning of the 1990s\textsuperscript{13} and in the past 4–5 years a trend towards a slight decrease has even been observed (Table 2). The level of resistance has not increased either, with 94% of highly PRP having an MIC of 2 mg/L. It is generally assumed that only pneumococci with a high level of resistance to penicillin can cause problems in the treatment of pneumococcal infections, so these strains must be carefully controlled.\textsuperscript{14} The data presented here suggest that β-lactams at correct doses can be used for the treatment of pneumococcal diseases in Spain.\textsuperscript{15,16} Resistance to fluoroquinolones, as in the rest of the world, was very low (1.5%), but these drugs must be used with caution because selection of resistance after treatment is not a rare finding, and an increasing tendency to resistance has already been observed in the USA and Spain.\textsuperscript{17–19}

The proportion of PRA-STs among PRP as a group has not differed significantly over time, representing \(\sim\)80–85% of PRP in each period, except for the 1979–1981 period (Table 3). However, as can be seen in Figure 2(b), the evolution of PRP within each particular PRA-ST has been very different. Spread of the well-established 6B and especially the 23F resistant clones and, on the other hand, the sudden emergence of clones 9V and 14 (Figure 2) explain the situation in Spain during the 1980s, when penicillin resistance increased from 6% to 44%.\textsuperscript{14} In this sense, the increase in serotype 23 pneumococci at the beginning of the 1980s was a striking example of

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure3.png}
\caption{(a) Evolution of serotypes 11, 15, 21 and 35 since 1979. (b) Proportion of penicillin-resistant strains within each serotype over time.}
\end{figure}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{MIC (mg/L)} & \textbf{No. of strains} & \textbf{Serotypes (no. of strains)} \\
\hline
8 & 8 & AT\textsuperscript{*}(8) \\
4 & 75 & 6 (12), 9 (11), 14 (13), 15 (2), 19 (4), 23 (10), AT (19), NT (4) \\
2 & 1333 & 6 (222), 9 (300), 14 (387), 15 (27), 16 (1), 19 (105), 23 (238), 35 (1), 40 (1), AT (33), NT (18) \\
\hline
\end{tabular}
\caption{Serotypes of highly penicillin-resistant pneumococci}
\end{table}

\textsuperscript{*}AT, atypical strains (see Table 3).
evolutionary change. In 1986 this serotype represented 18.4% of all the pneumococci studied, a figure never reached by any other serotype. Moreover, many of the penicillin-resistant serotypes 14 and 19 represent a variant of the 23F clone. All these data suggest that the Spanish 23F clone might have a special capacity for acquiring foreign DNA, and for colonization and transmission within the nasopharynx of children. Nevertheless, without any apparent change in antibiotic policy, the 23F clone has diminished in relation to the other PRA-STs, at present accounting for 9.7% of pneumococci.

Because penicillin resistance is confined to a small number of serotypes, most studies analysed only the prevalence of penicillin resistance associated with serotypes 6, 9, 14, 19 and 23, and no attention is focused on other minor penicillin-resistant serotypes. If, as expected, the circulation of PRA-STs among carriers declines after vaccination, other non-vaccine serotype PRPs might become more frequent within the nasopharynx, causing no change in resistance rates.

It is not possible to predict which serotypes will become predominant in the future if vaccine serotypes disappear as the main colonizers of the nasopharynx of children, but, as shown in Table 3, some serotypes have demonstrated their capacity for acquiring and maintaining resistance. It is noteworthy that PRP of serotypes other than PRA-STs were already present in Spain more than 20 years ago (Table 3), although they have remained uncommon over time. As shown in Figure 3, serotypes 15 and 35 are relatively frequent among pneumococci isolated in recent years and more than half of them are resistant to penicillin, so they could replace previously dominant serotypes in the future. In order to control the effect of the conjugate vaccine on the pneumococcal population, it is clear that the serotypes and the resistance of the pneumococci isolated in Spain should be monitored carefully.

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References

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