Real-time PCR per a la vigilància epidemiològica de la malaltia pneumocòccica invasiva (MPI) en pacients pediàtrics

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Tesi Doctoral

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ANNEX

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ARTICLE

Serotype 3 is a common serotype causing invasive pneumococcal disease in children less than 5 years old, as identified by real-time PCR

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Abstract Serotype 3 is one of the most often detected pneumococcal serotypes in adults and it is associated with serious disease. In contrast, the isolation of serotype 3 by bacterial culture is unusual in children with invasive pneumococcal disease (IPD). The purpose of this study was to learn the serotype distribution of IPD, including culture-negative episodes, by using molecular methods in normal sterile samples. We studied all children <5 years of age with IPD admitted to two paediatric hospitals in Catalonia, Spain, from 2007 to 2009. A sequential real-time polymerase chain reaction (PCR) approach was added to routine methods for the detection and serotyping of pneumococcal infection. Among 257 episodes (219 pneumonia, 27 meningitis, six bacteraemia and five others), 33.5% were identified by culture and the rest, 66.5%, were detected exclusively by real-time PCR. The most common serotypes detected by culture were serotypes 1 (26.7%) and 19A (25.6%), and by real-time PCR, serotypes 1 (19.8%) and 3 (18.1%). Theoretical coverage rates by the PCV7, PCV10 and PCV13 vaccines were 10.5, 52.3 and 87.2%, respectively, for those episodes identified by culture, compared to 5.3, 31.6 and 60.2% for those identified only by real-time PCR. Multiplex real-time PCR has been shown to be useful for surveillance studies of IPD. Serotype 3 is underdiagnosed by culture and is important in paediatric IPD.

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Introduction

Streptococcus pneumoniae is an important pathogen responsible for high mortality and morbidity worldwide, despite the availability of antibiotic treatment and vaccines [1]. Knowledge of pneumococci serotype distribution causing invasive pneumococcal disease (IPD) remains of primary importance in order to assess the effectiveness of new conjugate vaccines and closely monitor the emergence of non-vaccine serotypes.

Classic diagnosis of the disease is by microbiological bacterial culture, which has high specificity but low sensitivity, particularly in paediatric patients who have undergone prior antibiotic therapy; this increases the number of false-negative results [2]. Microbiological culture requires the isolation and identification of *S. pneumoniae* from normally sterile clinical specimens; it requires 48–72 h to confirm the results, which may have low sensitivity. Studies of serotype distribution based on

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culture-proved IPD have the same limitation. Therefore, antibiotic-susceptible pneumococcal serotypes may be misdiagnosed and, consequently, the rates of IPD can be underestimated. In addition, technical difficulties inherent in conventional serotyping limit its use to a few specialised laboratories. Different authors have reported that the sensitivity of molecular methods was significantly higher than culture methods, and that molecular methods can be used directly on sterile biological samples, improving the ability to diagnose IPD [3–8]. In addition, real-time PCR methods that specifically identify the capsular type in a direct sample offer a sensitive, rapid and simple approach for the surveillance of pneumococcal disease.

The objective of this study was to learn the serotype distribution, including culture-negative episodes of IPD, among young children before the introduction of 10-valent and 13-valent pneumococcal conjugate vaccines (PCV10 and PCV13) in Catalonia, Spain.

Materials and methods

Patients and setting

We studied all children <5 years of age with IPD who had been admitted to two tertiary-care paediatric hospitals in Barcelona from January 2007 to December 2009. Children included in the study have been analysed by routine microbiological methods and a sequential real-time PCR approach applied in normal sterile samples for the diagnosis and serotyping of IPD.

In Spain, the 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in June 2001. However, it was not included in the routine childhood vaccination schedule because it was not subsidised by the Spanish Health Service. The recommendations of the Spanish Paediatric Academy for the use of PCV7 were to cover all children aged <23 months and children aged 24–59 months who were at high risk for pneumococcal infection. The Academy recommends PCV7 vaccination for children aged ≤2 years, scheduled at 2, 4 and 6 months of age, with a booster in the second year of life, and for older children at high risk of IPD. During the study period, the use of PCV7 in the community was around 50% [9]. PCV10 and PCV13 were not introduced in our country during the study period.

IPD was defined as the presence of clinical findings of infection (which were used for the classification of disease), together with the isolation of *S. pneumoniae* and/or DNA detection of the *pneumolysin* (*ply*) gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any sterile fluid (plasma, cerebrospinal fluid or any other sterile fluid). DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR was performed according to a published assay [3] and was performed in the first 48 h after admission. IPD was classified according to the International Classification of Disease, Ninth Revision (ICD-9-CM) specific for diseases caused by *S. pneumoniae*, including: meningitis, pneumonia, parapneumonic empyaema, occult bacteraemia, sepsis and arthritis. Meningitis was considered by a compatible clinical syndrome and biochemical cerebrospinal fluid (CSF) test. Pneumonia was considered by the increase in respiratory rate, difficulty in breathing and pathological breath sounds. Complicated pneumonia was considered when a lobar or segmental lung consolidation with pleural effusion was detected. Occult bacteraemia/sepsis was considered among admitted patients with fever (>37.5°C axillary temperature), with or without clinical signs of sepsis. Osteomyelitis or arthritis were considered by the presence of local signs and confirmed by X-ray.

We registered the demographic and clinical variables, including: age, sex, date of admission, clinical manifestations, outcomes, vaccination status and previous antibiotic therapy (defined as exposure to an antibiotic treatment in the preceding 30 days before the diagnosis of IPD).

Data were recorded following the guidelines of the Hospital's Ethical Committee.

Microbiological culture and antimicrobial susceptibility studies of *S. pneumoniae* isolates

All pneumococcal isolates were identified by standard microbiological methods. The agar dilution technique was used to determine the minimum inhibitory concentration (MIC) of several antibiotics. Penicillin and other antibiotic susceptibilities were defined according to the breakpoints of the Clinical Laboratory Standards Institute (CLSI, M100-S20, 2010) [10].

Molecular diagnosis of *S. pneumoniae*

DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR in normal sterile samples was carried out according to a published assay [3] and was performed in the first 48 h after admission. The presence of *S. pneumoniae* DNA was confirmed by the amplification of the *wzg* (*CpsA*) gene by real-time PCR, as previously reported [11]. Only positive samples for both the *ply* and *wzg* genes in real-time PCR were included in the sequential serotyping analysis.

Serotype identification from direct clinical samples and *S. pneumoniae* isolates

The detection of pneumococcal serotypes from direct samples and from *S. pneumoniae* strains was performed at our laboratory, according to a published multiplex real-time PCR methodology [11]. This sequential PCR approach

detected 24 serotypes (1, 3, 4, 5, 6A/B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A/F).

In addition, strains isolated by culture were also serotyped using the Quellung reaction or dot blot. MICs and serotyping of the strains was performed at the National Center for Microbiology (Majadahonda, Spain).

Statistical analysis

We used the Chi-square test or Fisher's exact test, when appropriate, to compare proportions. Statistical analyses were performed using SPSS for Windows, version 17.0. We calculated 95% confidence intervals (CIs), and two-sided *p*-values ≤0.05 were considered to be statistically significant.

Results

During the study period, there were 319 patients with IPD, including 170 male patients (53.3%) and 149 female patients (46.7%), with a mean age of 29.6 months ± 15.7 months. Ninety-one (28.5%) patients had received antibiotic treatment in the month before the diagnosis of IPD and 168 (52.8%) children had received at least one dose of PCV7, although only 141 (44.3%) had been correctly vaccinated according to their age.

We included in the present study data of 257 serotyped episodes (80.6%) which had undergone both bacterial cultures and real-time PCR (we excluded 35 patients studied only by culture, nine studied only by real-time PCR and 18 without serotype study). One hundred and forty children (54.5%) were male and 117 (45.5%) were female, with a mean age of 30.95 months±15 months (age range from 20 days to 59 months). The distribution of patients by age group was as follows: 10 patients (3.8%) <6 months, 19 patients (7.4%) between 6–11 months, 64 patients (25%) between 12–23 months and 164 patients (63.8%) between 24–59 months. Eighty-three patients (32%) had received antibiotic treatment in the month before the diagnosis of IPD. One hundred and forty (54.5%) patients had received one dose or more of PCV7 and 122 (47.5%) were correctly vaccinated according their age.

Seventy-one (27.6%) patients had both positive culture and real-time PCR, 15 (5.8%) had positive culture and negative real-time PCR, and 171 (66.5%) had positive real-time PCR and negative culture. The proportion of cases diagnosed by real-time PCR only was 66.5% (95% CI 60.9–72.5), in contrast to 33.4% (95% CI 27.9–39.4) diagnosed by bacterial culture. The 257 episodes were detected in 64 (24.9%) positive blood specimens, 163 (63.4%) positive pleural fluid specimens, 28 (10.9%) positive CSF specimens and 2 (0.8%) positive joint fluid specimens. Table 1 shows the distribution of positive samples detected by culture and by real-time PCR only.

Overall, the clinical diagnosis of patients included in this study was pneumonia 219 (85.2%), meningitis 27 (10.5%), bacteraemia 6 (2.3%), arthritis 2 (0.8%), sepsis 2 (0.8%) and cellulitis 1 (0.4%). One hundred and eighty-eight (85.8%) of 219 patients with pneumonia had complicated pneumonia with empyaema. There were 4 (1.5%) deaths, comprising three patients with meningitis and one with sepsis. These episodes were caused by serotypes 7F, 27, 6A and 23F (this last one occurring in an unvaccinated child).

The major increase of microbiological diagnosis by using real-time PCR was in patients with pneumonia; 74.0% (95% CI 68.4–80.0) were only diagnosed by real-time PCR, while 26% (95% CI 20.4–32.0) were diagnosed by bacterial culture. Statistically significant differences were also observed in meningitis and bacteraemia (Table 2).

Serotyping study was done in 86 (33.5%) strains isolated from culture and the remaining 171 (66.5%) episodes directly by real-time PCR from the biological sample.

Only two of the 86 strains (2.3%) with available antimicrobial susceptibility study were penicillin intermediate-resistant (MIC 4 μg/mL) and none (0%) penicillin fully-resistant according to non-meningeal breakpoints. The percentage of penicillin non-susceptible isolates was 33.7% and cefotaxime 16.3% according to meningeal breakpoints, and the serotypes that caused the most penicillin non-susceptible-related IPDs were serotype 19A (51.7% of non-susceptible isolates), serotype 23B (10.3%) and serotype 24B/F (10.3%).

Differences in serotype distribution among patients with positive culture versus patients with negative culture

Fifty percent of patients identified by culture and 48% of patients identified only by real-time PCR had been well vaccinated with PCV7. No patients were vaccinated with either PCV10 or PCV13. We found significant differences ($p<0.002$) in the rank order of the five main serotypes in IPD episodes identified by culture versus. those identified only by real-time PCR. The three most frequent serotypes in the group of 86 episodes identified by culture were serotype 1 (28%; $n=23$), serotype 19A (26%; $n=22$) and serotype 7F (9%; $n=8$), while in the group of 171 patients diagnosed only by real-time PCR, they were serotype 1 (20%; $n=34$), serotype 3 (18%; $n=31$) and serotype 19A (9%; $n=16$). Of note, the rate of serotype 3 detected by real-time PCR was significantly higher than the rate of this serotype detected by culture ($p=0.01$). Figure 1 shows the different serotype distributions according to diagnosis by culture or only real-time PCR.

As expected for the routine use of PCV7 during the study period, IPD caused by serotypes included in PCV7 was a rare event. PCV7 serotypes were found in 10.5% of patients identified by culture versus 5.3% of patients identified by real-time PCR ($p=0.12$). Among the PCV7

Table 1 Distribution of positive samples detected by culture and only by real-time polymerase chain reaction (PCR)

Type of sample	Positive samples by culture, *n* (%[a]; 95% CI)	Positive samples only by real-time PCR, *n* (%[a]; 95% CI)	*p*-value[b]
Plasma	21 (32.8; 22.1–44.9)	43 (67.2; 55–77.8)	<0.001
Pleural fluid	42 (25.8; 19.5–32.9)	121 (74.2; 67.1–80.5)	<0.001
CSF	21 (75; 56.7–88.3)	7 (25; 11.6–43.3)	<0.001
Joint fluid	2 (100; 22.4–100)	0 (0; 0–77.6)	0.3

[a] Percentage with respect to all positive samples by type of sample

[b] Pearson's Chi-square or Fisher's exact test comparing percentage by type of sample

cases by culture (n=9 patients; 10.5%), only one child (11%) had received three doses of PCV7, and among the PCV7 cases by PCR (n=9 patients; 5.3%), three children (33.3%) had received one, two or three doses of PCV7. The proportion of serotypes included in the PCV10 rose to 52.3% in patients identified by culture versus 31.6% in patients detected only by real-time PCR (p<0.002). Serotypes included in PCV13 were detected in 87.2% of patients diagnosed by positive culture and in 60.2% of patients diagnosed by real-time PCR (p<0.000). Figure 2 shows the potential coverage of these three conjugate vaccines according to diagnosis by culture or only by real-time PCR.

Differences in serotype distribution according to age and microbiological diagnosis technique

Overall, patients identified by culture were significantly younger than patients identified only by real-time PCR (mean age 25.9 months ±15 months vs. 33.4 months ±14.1 months).

Of the total number of children studied, 93 (36.2%) were younger than 2 years of age; of these, 46 (49.5%; 95% CI 38.7–60.2) were identified by culture and 47 (50.5%; 95% CI 39.8–61.2) were identified only by real-time PCR. In the group of patients identified by culture, the main serotype detected was serotype 19A (16 patients; 34.8%), while in those identified only by real-time PCR, serotype 3 was the strain most often detected (14 patients; 29.8%).

Among children over 2 and less than 5 years of age (n=164), 40 were identified by culture (24.4%; 95% CI 17.5–31.3) and 124 (75.6%; 95% CI 68.7–82.5) were identified only by real-time PCR. Serotype 1 was the main serotype detected in patients identified by culture (20 patients; 50%) and by real-time PCR (32 patients; 25.8%). The second most prevalent serotype was serotype 19A among patients identified by culture (6 patients; 15%) and serotype 3 among patients identified by real-time PCR (17 patients; 13.7%).

Differences in serotype distribution among patients with pneumonia according to microbiological diagnosis technique

Among episodes of pneumonia, we found significant differences (p<0.001) in the serotype distribution of isolates when comparing episodes identified by culture versus those identified only by real-time PCR. Twenty-three of 57 episodes (68.4%) identified by culture were caused by serotypes 1 (40.3%) and 19A (28%). In this group, serotype 3 was found in only 5 patients (8.7%). However, in 162 episodes identified only by real-time PCR, the second most

Table 2 Clinical forms of invasive pneumococcal disease (IPD) in children diagnosed by bacterial culture versus those diagnosed only by real-time PCR

Clinical form	Patients diagnosed by culture, *n* (%[a]; 95% CI)	Patients diagnosed only by real-time PCR, *n* (%[a]; 95% CI)	*p*-value[b]
Pneumonia	57 (26.0; 20.4–32.0)	162 (74.0; 68.4–80.0)	<0.001
Meningitis	20 (74.1; 53.7–88.9)	7 (25.9; 11.1–46.3)	0.09
Bacteraemia	6 (100)	0	0.001
Sepsis	0	2 (100)	0.55
Arthritis	2 (100)	0	0.11
Cellulitis	1	0	0.33
Total	86 (33.4; 27.9–39.4)	171 (66.5; 60.9–72.5)	<0.001

[a] Percentage with respect to all microbiological diagnoses by clinical form

[b] Pearson's Chi-square or Fisher's exact test comparing the distribution of microbiological diagnoses in each clinical form with respect to all clinical forms

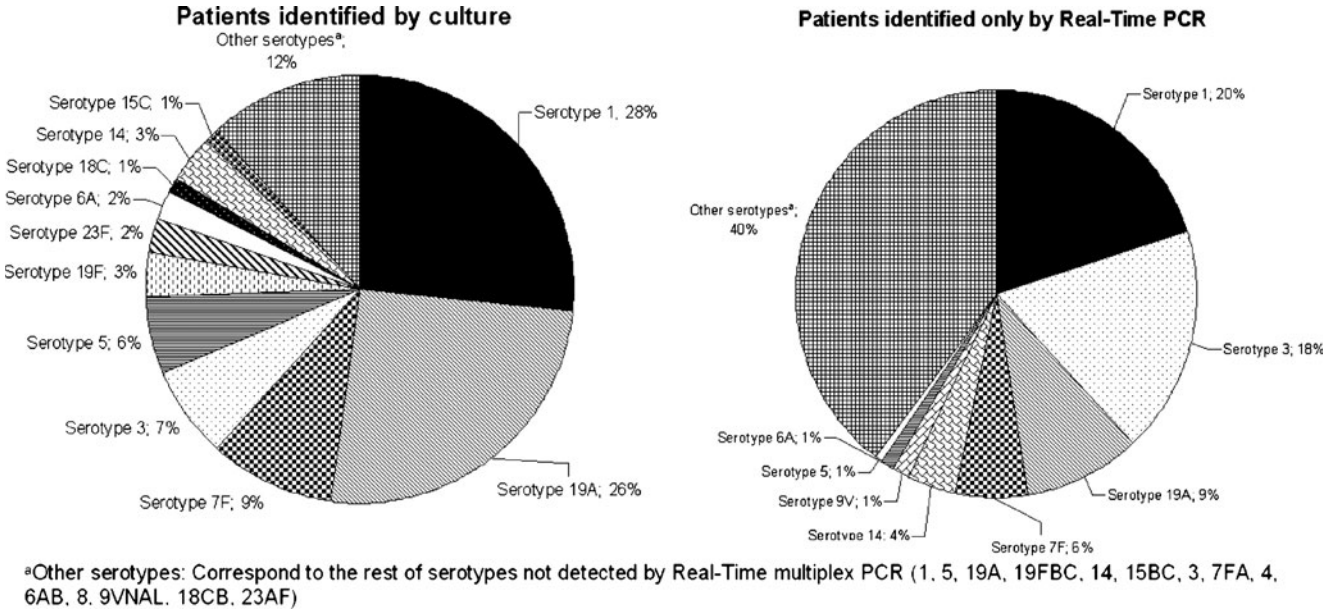

Fig. 1 Serotype distribution according to diagnosis by culture or only real-time polymerase chain reaction (PCR)

prevalent serotype causing IPD was serotype 3 (19.1%, 31 patients). Serotypes 1 and 19A were detected in 34 (20.9%) and 15 (9.2%) episodes, respectively.

Differences in serotype distribution according to previous antibiotic therapy and microbiological diagnosis technique

The microbiological study was performed after previous antibiotic therapy in 83 (32.3%) children. In this group of patients, only 13 (15.3%) showed positive results of bacterial culture, while in the group of 172 (66.9%) patients without previous antibiotic therapy, bacterial culture was positive in 72 (41.9%). This difference is statistically significant ($p<0.000$). For two patients, information about their previous antibiotic therapy was not available.

We detected important and significant differences ($p=0.02$) of the serotype distribution in patients exposed to previous antibiotic therapy according to the microbiological diagnosis technique (Table 3). Of the total patients with previous antibiotic therapy and a positive culture ($n=13$),

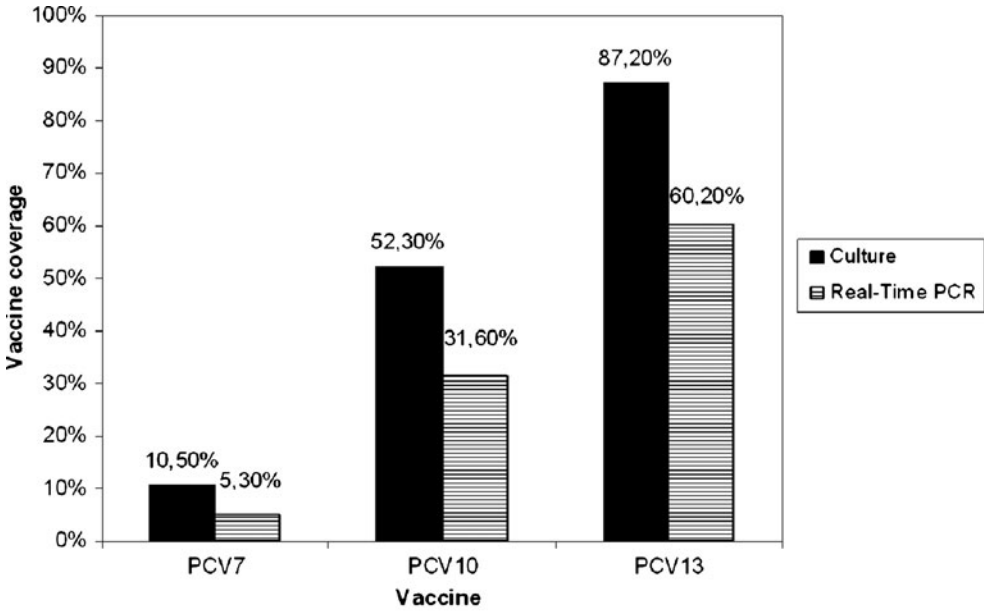

Fig. 2 Coverage of different pneumococcal conjugate vaccines (PCVs)

PCV7: Pneumococcal conjugate vaccine 7-valent, PCV10: Pneumococcal conjugate vaccine 10-valent, PCV13: Pneumococcal conjugate vaccine 13-valent

Table 3 Serotype distribution according to previous antibiotic therapy and microbiological diagnosis technique in 255 children[a] with IPD

Serotypes	Patients exposed to previous antibiotic therapy		Patients not exposed to previous antibiotic therapy	
	n (%)		*n* (%)	
	Patients diagnosed by culture	Patients diagnosed only by real-time PCR	Patients diagnosed by culture	Patients diagnosed only by real-time PCR
1	2 (15.4)	14 (20)	21 (29.2)	20 (20)
19A	6 (46.2)	7 (10)	16 (22.2)	9 (9)
5	2 (15.4)	1 (1.4)	3 (4.2)	0 (0.0)
3	0 (0.0)	12 (17.1)	6 (8.3)	19 (19)
7F	1 (7.6)	5 (7.1)	7 (9.7)	5 (5)
14	0 (0.0)	2 (2.9)	3 (4.2)	5 (5)
6A	0 (0.0)	1 (1.4)	2 (2.8)	1 (1)
19F	0 (0.0)	0 (0.0)	3 (4.2)	0 (0.0)
23F	0 (0.0)	0 (0.0)	2 (2.8)	0 (0.0)
9V	0 (0.0)	0 (0.0)	0 (0.0)	2 (2)
15C	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)
18C	0 (0.0)	0 (0.0)	1 (1.4)	0 (0.0)
Other serotypes[b]	2 (15.4)	28 (40)	7 (9.45)	39 (39)
Overall	13 (100)	70 (100)	72 (100)	100 (100)

[a] For two patients, information about their previous antibiotic therapy was not available

[b] Correspond to the rest of the serotypes not detected by real-time multiplex PCR (non-serotypes 1, 5, 19A, 19F/B/C, 14, 15B/C, 3, 7F/A, 4, 6A/B, 8, 9V/A/N/L, 18C/B, 23A/F)

the main serotype detected was serotype 19A (6 isolates; 46.2%). However, among the total group of patients exposed to antibiotic therapy and diagnosed by real-time PCR (n=70), the main serotype detected was serotype 1 (14 isolates, 20.0%).

In the group of patients not exposed to previous antibiotic therapy with positive culture (n=72 patients), serotype 1 was the most frequently detected serotype (21 isolates, 29.2%), followed by serotype 19A (16 isolates, 22.2%) and serotype 7F (7 isolates, 9.7%). In contrast, in the 100 episodes of patients without antibiotic exposure and diagnosed only by real-time PCR, the main serotypes detected were serotype 1 (20 isolates; 20%), followed by serotype 3 (19 isolates, 19%) and serotype 19A (9 isolates, 9%) (p=0.02).

Discussion

In the present study, performed during the routine use of PCV7 vaccine, we found that the proportion of microbiological diagnoses of IPD carried out only by real-time PCR (with negative culture) is twice the proportion of diagnoses carried out by culture. The diagnosis of *S. pneumoniae* infections may be problematic, mainly in paediatric children, who present the peculiarity that it is not always possible to collect an adequate volume of the sample on which to perform blood culture. Moreover, many of these patients received treatment with antibiotics previous to sample collection and, therefore, cultures are frequently negative. In this study, comparing the patients with previous antibiotic therapy, only 15.3% showed positive results of bacterial culture, in contrast to patients without previous antibiotic therapy, among whom 41.9% of the cases showed positive results to bacterial culture. For this reason, new sensitive diagnostic methods are needed not only for diagnosis, but also to monitor the epidemiology of pneumococcal disease and the impact of vaccines.

This study has value for epidemiologic surveillance and also as a further evaluation of the potential impact of new conjugate vaccines. Immunisation with PCV7 has changed the distribution of the main serotypes causing IPD [9, 12, 13]. In Spain, Fenoll et al. [14] reported the temporal trends of invasive *S. pneumoniae* serotypes and antimicrobial resistance over a period of 30 years; serotypes 1 and 19A have become more prevalent since the introduction of PCV7, while other serotypes, such as 3, 4 and 8, have maintained their steady secular trend over the three decades. Temporal trends of pneumococcal serotype distribution have been reported and observed during different periods of time [14–16] and have been associated with antibiotic treatment or/and vaccines.

Changes in serotype distribution may be an important factor to explain changes in the epidemiological characteristics of IPD comparing the pre-vaccine and vaccine eras. In the pre-vaccine era, the risk of IPD was usually highest in those <2 years old and then tapers off after 2 through 5 years of age. In the present study, children >2 years old are the main group with IPD. This fact was also detected in previous studies of our group, where bacterial culture was the only microbiological criterion for the definition of IPD [9]. The emergence of non-vaccine serotypes, such as serotype 1, which is mainly detected in older children, and the good results of PCV7 against serotypes mainly detected in children younger than 2 years of age, are partial explanations for this event.

Therefore, it is important to learn the distribution of serotypes in our population in order to analyse the impact of PCV7 before the introduction of the new conjugate vaccines (10-valent and 13-valent, PCV10 and PCV13, respectively). According to our results, the coverage of the current conjugate vaccines PCV7, PCV10 and PCV13 is lower than that expected in those patients diagnosed only by real-time PCR, especially in PCV7 and PCV10. For this reason, further molecular epidemiology studies are needed after vaccination in order to predict the trends in particular serotypes and to detect a possible replacement phenomenon for non-vaccine serotypes similar to that detected in PCV7.

The serotype distribution of patients identified by culture reported in this study is similar to what other authors have found [17, 18]. The most prevalent serotype in patients identified by culture is serotype 1, as in other regions of Spain and in Portugal. Marimon et al. [19] reported an increase in the number of IPD cases in children caused by serotype 1 in the Basque region of Spain following the introduction of PCV7. Nunes et al. [20] reported an emergence of serotype 1 lineage of pneumococci among healthy carriers in Portugal after 2003. The other most prevalent serotypes detected by culture were serotypes 19A and 7F. Serotype 19A has been reported by many authors as the most common serotype causing invasive pneumococcal infections in children [12, 21, 22], and many 19A isolates have been associated with multidrug resistance [23, 24]. A surveillance study conducted in Germany [25] beginning in 1992 reported that serotype 7F was statistically more prevalent among children less than 4 months old than among individuals in other age groups.

Prevalent serotypes found by culture have also been common among patients detected only by real-time PCR, but we have found additional serotypes identified only by real-time PCR that are not so commonly seen among culture episodes. In the rank order of serotypes in patients with negative culture, we found serotype 3 to be the second most frequent serotype. Serotype 3 has been associated with invasive disease in older children and adults [26] and with higher case–fatality ratios compared to other serotypes [27]. In Spain, a study reported that serotype 3 was one of the most prevalent serotypes causing paediatric parapneumonic empyaema (PPE) and was associated with significantly more complications than PPE caused by other serotypes [28]. Recently, Bender et al. [29] identified an increasing incidence of *S. pneumoniae*-related haemolytic uraemic syndrome in children in Utah, associated with serotype 3. We have found that serotype 3 is mainly detected by molecular methods and is less frequent in those patients identified by culture. According to this data, multiplex real-time PCR has the potential to reveal a different distribution of serotypes circulating in the population compared to culture-positive cases. A recent study comparing conventional and molecular microbiology in detecting differences in pneumococcal colonisation among healthy carriers and ill children showed that real-time PCR was superior to bacterial culture in identifying a great number of pneumococcal serotypes in both groups of patients, healthy nasopharyngeal carriers and children with upper respiratory illness [30].

Our study has several limitations. First, the real-time multiplex PCR [11] assay used in this study does not differentiate between certain serotypes, such as 6A/C or 19F/B/C, although it detects all serotypes included in the three conjugate vaccines. Second, we found that 44% of pneumococci in patients diagnosed by real-time PCR corresponded to serotypes other than those detected by real-time multiplex PCR. This could be explained by the fact that the number of serotypes detected in the assay was limited to 24 of the 93 serotypes currently known. Another putative explanation is that these pneumococci may be other species closely related to *S. pneumoniae*, rather than *S. pneumoniae* itself. Recently, new species such us *Streptococcus pseudopneumoniae* [31, 32] and closely related streptococci [33] have been described in the literature. In our laboratory, we perform real-time PCR of the *pneumolysin* (*ply*) gene as screening and a second real-time PCR assay to detect the capsular *wzg* gene before performing the serotyping study. Therefore, the detection of a virulence gene of pneumococci and an additional capsular gene in a sterile sample of a patient with clinical symptoms of bacterial infection may have significant clinical value. Although few data are available, it has been reported [33] that these closely related streptococci are critical in pneumococcal colonisation studies because they inhabit the same niche and can be highly resistant to antibiotics. The clinical role of these closely related pneumococcal strains isolated in sterile samples needs to be clarified.

The results of this study may be different from those obtained in other geographical areas, with different use of PCV7, different antibiotic political use or different use of

blood cultures. The present study is performed with a intermediate introduction of PCV7 (around 50%), intermediate use of previous antibiotics (32% of children were exposed to antibiotics) and only including hospitalised patients. In addition, the presence of a specific clone such as ST306 of serotype 1 in our community may be related with the high prevalence of pneumonia [34] and disease observed in older healthy children, while in other communities with other predominant clones (i.e multiresistant PCV7 serotypes), the picture of disease may be different.

To conclude, multiplex real-time PCR has been shown to be very useful for surveillance studies of IPD and it is a good complement for classical microbiological methods. Serotype 3 is underdiagnosed by culture and it is important in paediatric IPD.

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References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N et al (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 374:893–902
2. Zhang Y, Isaacman DJ, Wadowsky RM, Rydquist-White J, Post JC, Ehrlich GD (1995) Detection of *Streptococcus pneumoniae* in whole blood by PCR. J Clin Microbiol 33:596–601
3. Muñoz-Almagro C, Gala S, Selva L, Jordan I, Tarragó D, Pallares R (2011) DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema. Eur J Clin Microbiol Infect Dis 30:327–335
4. Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, de Martino M et al (2008) Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. J Med Microbiol 57:1205–1212
5. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB (2001) Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 39:1553–1558
6. Van Gastel E, Bruynseels P, Verstrepen W, Mertens A (2007) Evaluation of a real-time polymerase chain reaction assay for the diagnosis of pneumococcal and meningococcal meningitis in a tertiary care hospital. Eur J Clin Microbiol Infect Dis 26:651–653
7. Lahti E, Mertsola J, Kontiokari T, Eerola E, Ruuskanen O, Jalava J (2006) Pneumolysin polymerase chain reaction for diagnosis of pneumococcal pneumonia and empyema in children. Eur J Clin Microbiol Infect Dis 25:783–789
8. Picazo J, Ruiz-Contreras J, Casado-Flores J, Giangaspro E, Del Castillo F, Hernández-Sampelayo T et al (2011) Relationship between serotypes, age, and clinical presentation of invasive pneumococcal disease in Madrid, Spain, after introduction of the 7-valent pneumococcal conjugate vaccine into the vaccination calendar. Clin Vaccine Immunol 18:89–94
9. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, García-García JJ, Pallares R (2008) Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 46:174–182
10. Clinical and Laboratory Standards Institute (CLSI) (2010) Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement. M100-S20. CLSI, Wayne, PA, USA
11. Tarragó D, Fenoll A, Sánchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C et al (2008) Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin Microbiol Infect 14:828–834
12. Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA et al (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 297:1784–1792
13. Lehmann D, Willis J, Moore HC, Giele C, Murphy D, Keil AD et al (2010) The changing epidemiology of invasive pneumococcal disease in aboriginal and non-aboriginal western Australians from 1997 through 2007 and emergence of nonvaccine serotypes. Clin Infect Dis 50:1477–1486
14. Fenoll A, Granizo JJ, Aguilar L, Giménez MJ, Aragoneses-Fenoll L, Hanquet G et al (2009) Temporal trends of invasive *Streptococcus pneumoniae* serotypes and antimicrobial resistance patterns in Spain from 1979 to 2007. J Clin Microbiol 47:1012–1020
15. Siira L, Rantala M, Jalava J, Hakanen AJ, Huovinen P, Kaijalainen T et al (2009) Temporal trends of antimicrobial resistance and clonality of invasive *Streptococcus pneumoniae* isolates in Finland, 2002 to 2006. Antimicrob Agents Chemother 53:2066–2073
16. Harboe ZB, Benfield TL, Valentiner-Branth P, Hjuler T, Lambertsen L, Kaltoft M et al (2010) Temporal trends in invasive pneumococcal disease and pneumococcal serotypes over 7 decades. Clin Infect Dis 50:329–337
17. Byington CL, Hulten KG, Ampofo K, Sheng X, Pavia AT, Blaschke AJ et al (2010) Molecular epidemiology of pediatric pneumococcal empyema from 2001 to 2007 in Utah. J Clin Microbiol 48:520–525
18. Weatherholtz R, Millar EV, Moulton LH, Reid R, Rudolph K, Santosham M et al (2010) Invasive pneumococcal disease a decade after pneumococcal conjugate vaccine use in an American Indian population at high risk for disease. Clin Infect Dis 50:1238–1246
19. Marimon JM, Ercibengoa M, Alonso M, Zubizarreta M, Pérez-Trallero E (2009) Clonal structure and 21-year evolution of *Streptococcus pneumoniae* serotype 1 isolates in Northern Spain. Clin Microbiol Infect 15:875–877
20. Nunes S, Sá-Leão R, Pereira LC, Lencastre H (2008) Emergence of a serotype 1 *Streptococcus pneumoniae* lineage colonising healthy children in Portugal in the seven-valent conjugate vaccination era. Clin Microbiol Infect 14:82–84
21. Kaplan SL, Barson WJ, Lin PL, Stovall SH, Bradley JS, Tan TQ et al (2010) Serotype 19A is the most common serotype causing

invasive pneumococcal infections in children. Pediatrics 125:429–436
22. Muñoz-Almagro C, Esteva C, de Sevilla MF, Selva L, Gene A, Pallares R (2009) Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. J Infect 59:75–82
23. Moore MR, Gertz RE Jr, Woodbury RL, Barkocy-Gallagher GA, Schaffner W, Lexau C et al (2008) Population snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States, 2005. J Infect Dis 197:1016–1027
24. Reinert RR, Reinert S, van der Linden M, Cil MY, Al-Lahham A, Appelbaum P (2005) Antimicrobial susceptibility of *Streptococcus pneumoniae* in eight European countries from 2001 to 2003. Antimicrob Agents Chemother 49:2903–2913
25. Imöhl M, Reinert RR, Ocklenburg C, van der Linden M (2010) Association of serotypes of *Streptococcus pneumoniae* with age in invasive pneumococcal disease. J Clin Microbiol 48:1291–1296
26. Hausdorff WP, Bryant J, Paradiso PR, Siber GR (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis 30:100–121
27. Rückinger S, von Kries R, Siedler A, van der Linden M (2009) Association of serotype of *Streptococcus pneumoniae* with risk of severe and fatal outcome. Pediatr Infect Dis J 28:118–122
28. Obando I, Muñoz-Almagro C, Arroyo LA, Tarrago D, Sanchez-Tatay D, Moreno-Perez D et al (2008) Pediatric parapneumonic empyema, Spain. Emerg Infect Dis 14:1390–1397
29. Bender JM, Ampofo K, Byington CL, Grinsell M, Korgenski K, Daly JA et al (2010) Epidemiology of *Streptococcus pneumoniae*-induced hemolytic uremic syndrome in Utah children. Pediatr Infect Dis J 29:712–716
30. Ogami M, Hotomi M, Togawa A, Yamanaka N (2010) A comparison of conventional and molecular microbiology in detecting differences in pneumococcal colonization in healthy children and children with upper respiratory illness. Eur J Pediatr 169:1221–1225
31. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, Steigerwalt AG et al (2004) Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J Clin Microbiol 42:4686–4696
32. Keith ER, Podmore RG, Anderson TP, Murdoch DR (2006) Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. J Clin Microbiol 44:923–927
33. Simões AS, Sá-Leão R, Eleveld MJ, Tavares DA, Carriço JA, Bootsma HJ et al (2010) Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol 48:238–246
34. Muñoz-Almagro C, Selva L, Sanchez CJ, Esteva C, de Sevilla MF, Pallares R et al (2010) PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia and is strongly associated with clonal type. Clin Vaccine Immunol 17:1672–1678

advisories and travel health precautions were subsequently released, including those from Canada and United States (*9*,*10*). These precautions recommended that preventative measures such as vaccination and safe food and water consumption practices be adhered to by residents and visitors to affected regions. Although the public health community anticipated that travel-associated cases would be diagnosed in Québec, this report of a documented case (supported by laboratory and epidemiologic data) emphasizes the domestic and international public health risk caused by the nationwide outbreak in Haiti. It also illustrates the need for an accurate travel history in clinical and laboratory diagnosis of cholera infections.

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References

1. Pan American Health Organization. PAHO responds to cholera outbreak on the island of Hispaniola; 2011 [cited 2011 Mar 28]. http://new.paho.org/disasters/index.php?option=com_content&task=view&id=1423&Itemid=1
2. Clinical and Laboratory Standards Institute (CLSI). Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria. M45A2. Wayne (PA): The Institute; 2006.
3. Public Health Agency of Canada. Laboratory surveillance data for enteric pathogens in Canada. Annual summary 2006 [cited 2011 Mar 28]. http://www.nml-lnm.gc.ca/NESP-PNSME/assets/pdf/2006AnnualReport.pdf
4. Centers for Disease Contro land Prevention. Update: outbreak of cholera—Haiti, 2010. MMWR Morb Mortal Wkly Rep. 2010;59:1473–9.
5. Chin CS, Sorenson J, Harris JB, Robins WP, Charles RC, Jean-Charles RR, et al. The origin of the Haitian cholera outbreak strain. N Engl J Med. 2011;364:33–42. doi:10.1056/NEJMoa1012928
6. Swaminathan B, Gerner-Smidt P, Ng LK, Lukinmaa S, Kam KM, Rolando S, et al. Building PulseNet International: an interconnected system of laboratory networks to facilitate timely public health recognition and response to foodborne disease outbreaks and emerging foodborne diseases. Foodborne Pathog Dis. 2006;3:36–50. doi:10.1089/fpd.2006.3.36
7. Staley C, Harwood VJ. The use of genetic typing methods to discriminate among strains of *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. J AOAC Int. 2010;93:1553–69.
8. Cooper KL, Luey CK, Bird M, Terajima J, Nair GB, Kam KM, et al. Development and validation of a PulseNet standardized pulsed-field gel electrophoresis protocol for subtyping of *Vibrio cholerae*. Foodborne Pathog Dis. 2006;3:51–8. doi:10.1089/fpd.2006.3.51
9. Update on travel to Haiti: cholera outbreak [cited 2011 Mar 28]. http://www.phac-aspc.gc.ca/tmp-pmv/thn-csv/quake-tremble-haiti-eng.php
10. Centers for Disease Control and Prevention. Cholera in Haiti: CDC travelers' health [cited 2011 Mar 28]. http://www-wnc.cdc.gov/travel/content/travel-health-precaution/haiti-cholera.aspx

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Easy Diagnosis of Invasive Pneumococcal Disease

To the Editor: Invasive pneumococcal disease (IPD) causes many cases of severe disease and death among children <5 years of age, mostly in developing countries (*1*,*2*). Before conjugate vaccines can be introduced in developing countries, information about disease epidemiology is urgently needed. The lack of laboratories equipped to perform pneumococcal serotyping leads to the need to send isolates to reference laboratories. Good sample preservation is necessary to prevent samples from arriving at the laboratory in poor condition. We evaluated the usefulness of multiplex real-time PCR from strains and blood samples kept at room temperature on dried blood spot (DBS) filter paper for detecting and serotyping *Streptococcus pneumoniae*. DBS screening is a reliable method that requires only a small amount of blood; it is used for the diagnosis of several human diseases (*3*,*4*).

To validate the technique, we selected 15 pneumococcus clinical isolates representing 15 serotypes (1, 5, 19A, 19F, 14, 3, 7F, 4, 6A, 6B, 8, 9N, 18C, 23A, 23F) obtained during 2009 from patients at Hospital Sant Joan de Déu, in Barcelona. These isolates, used as controls, had been serotyped by quellung reaction at the Instituto de Salud Carlos III, Majadahonda-Madrid, Spain. These strains were cultured overnight at 35°C in 5% carbon dioxide on Columbia agar plates with 5% sheep blood (bioMérieux SA, Marcy l'Etoile, France). A suspension of each strain was adjusted to match a 0.5 McFarland standard (equivalent to 10^8 colony-forming units (CFU)/mL). Stock solutions of pneumococcus culture for each previously identified serotype were injected into blood

previously extracted from 2 healthy volunteers. Serial dilutions of 100,000 CFU/mL to 1,000 CFU/mL (1,000 to 10 CFU equivalents/PCR) were performed. A total of 100 µL of blood was applied to DBS filter paper, and another 100 µL was used for DNA extraction from fresh blood. All DBS samples were air dried for 1 week. The procedure was also performed on negative control blood samples.

DNA was extracted from DBS and fresh blood samples by using the NucliSense easyMAG automated extraction platform (bioMérieux, Boxtel, the Netherlands) according to the manufacturer's instructions. DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR was performed according to a published assay (*5*). In addition, we performed a multiplex real-time PCR for molecular serotype detection of serotypes 1, 3, 5, 4, 6A, 6B, 7FA, 8, 9VANL, 14, 15BC, 18CB, 19A, 19FBC, 23F, 23A and the conserved capsular gene *wzg* as described by Tarrago et al. (*6*). DNA extracts were amplified with the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). Negative results were defined as those with cycle threshold >40.

To evaluate the reliability obtained with this in vivo approach, we performed identification and serotyping of *S. pneumoniae* in 25 DBS samples from 25 children at Saint John of God Hospital in Mabesseneh-Lunsar, Sierra Leone. This hospital does not perform blood cultures. IPD was confirmed when DNA of a *pneumolysin (ply)* gene and an additional capsular gene of *S. pneumoniae* were detected by multiplex real-time PCR of DBS samples.

Detection of *ply*, *wzg*, and the specific gene for molecular serotype showed that both fresh blood and DBS samples yielded correctly positive results from the 10-fold serial dilutions analyzed (Table). With respect to the 25 (11 female and 14 male) patients from Sierra Leone who had suspected IPD, the median age was 25.71 months (range 15 days to 96 months); all had a diagnosis of fever without apparent source, and 16 also had malaria. Of these 25 children, DBS samples from 15 (60%) yielded a positive result for the *ply* and *wzg* genes, so they were considered confirmed episodes of IPD. A serotype included in 13-valent conjugate vaccine was detected in 6 (40%) of 15 positive samples: serotypes 3, 7FA, 19A, 6A, 6B, and 9VNL (1 sample each). In the remaining 9 samples, the results for *ply* gene and *wzg* gene were positive, but none of the 24 tested serotypes was detected.

This preliminary study enabled us to demonstrate that DBS screening is a reliable and easy method for diagnosing IPD and also for epidemiologic surveillance of the more frequent serotypes. The main limitation of our study is the small number of DBS samples sent from Saint John of God Hospital in Sierra Leone.

In conclusion, the DBS technique enables reproducible transport of samples for identification and serotyping of *S. pneumoniae* by multiplex PCR. The use of DBS on filter paper is an attractive alternative method for storing samples at room temperature and easily transporting them. Additional studies, including evaluation of the relative sensitivity of this method compared to direct culture, are necessary.

Table. Sensitivity of real-time PCR for detecting *Streptococcus pneumoniae ply* or *wzg* genes or a specific gene for molecular serotype from fresh or dried blood spot samples*

	Serial dilutions correctly detected, CFU equivalent/PCR†	
Gene or serotype	Fresh blood	Dried blood spot
ply gene	1.10^1–1.10^3	1.10^1–1.10^3
wzg gene	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 1	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 5	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 19A	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 19F	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 14	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 3	**1.10^2–1.10^3**	1.10^1–1.10^3
Serotype 7F	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 4	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 6A	1.10^1–1.10^3	**1.10^2–1.10^3**
Serotype 6B	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 8	1.10^1–1.10^3	1.10^2–1.10^3
Serotype 9N	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 18C	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 23A	1.10^1–1.10^3	1.10^1–1.10^3
Serotype 23F	1.10^1–1.10^3	1.10^1–1.10^3

*Serial dilutions (10–1,000 CFU equivalent/PCR) of 15 pneumococcus cultures mixed with 15 negative-control blood samples were analyzed. **Boldface** indicates results that differ from others.
†10 CFU equivalent/PCR = 1,000 CFU equivalents/mL blood.

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References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet. 2009;374:893–902. doi:10.1016/S0140-6736(09)61204-6
2. Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H. Epidemiology and etiology of childhood pneumonia. Bull World Health Organ. 2008;86:408–16. doi:10.2471/BLT.07.048769
3. Boppana SB, Ross SA, Novak Z, Shimamura M, Tolan RW Jr, Palmer AL, et al. Dried blood spot real-time polymerase chain reaction assays to screen newborns for congenital cytomegalovirus infection. JAMA. 2010;303:1375–82. doi:10.1001/jama.2010.423
4. De Crignis E, Re MC, Cimatti L, Zecchi L, Gibellini D. HIV-1 and HCV detection in dried blood spots by SYBR Green multiplex real-time RT-PCR. J Virol Methods. 2010;165:51–6. Epub 2010 Jan 5. doi:10.1016/j.jviromet.2009.12.017
5. Muñoz-Almagro C, Gala S, Selva L, Jordan I, Tarragó D, Pallares R. DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema. Eur J Clin Microbiol Infect Dis. 2010 Oct 24.
6. Tarragó D, Fenoll A, Sánchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin Microbiol Infect. 2008;14:828–34. doi:10.1111/j.1469-0691.2008.02028.x

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Mimivirus-like Particles in Acanthamoebae from Sewage Sludge

To the Editor: Mimivirus is a giant, double-stranded DNA virus. Its 650-nm diameter and 1.2-Mb genome make it the largest known virus (*1*). In 2003, mimivirus was isolated from a water cooling tower in Bradford, UK, after a pneumonia outbreak and was reported to infect *Acanthamoeba polyphaga* amebae (*2*). Subsequently, a small number of additional isolates have been reported (*3*).

Mimivirus has been associated with pneumonia, and this association was strengthened after antibodies to mimivirus were found in serum samples from patients with community- and hospital-acquired pneumonia and after mimivirus DNA was found in bronchoalveolar lavage specimens (*4*). More direct evidence of pathogenicity was illustrated when a pneumonia-like disease developed in a laboratory technician who worked with mimivirus and showed seroconversion to 23 mimivirus-specific proteins (*5*).

We report finding mimivirus-like particles during our molecular study of *Acanthamoeba* spp. abundance and diversity in final-stage conventionally treated sewage sludge from a wastewater treatment plant in the West Midlands, UK. Using metagenomic DNA extracted from the sludge (*6*), we estimated the abundance of *Acanthamoeba* spp. by using real-time PCR (*7*) and found it to be ≈1 × 10^2/g sludge. To assess species diversity, we amplified an *Acanthamoeba* spp.–specific 18S rRNA target, which resulted in products of ≈450 bp (*8*). PCR products were cloned and sequenced, revealing low *Acanthamoeba* spp. diversity with a predominance of clones most similar to *A. palestinensis* (22/25 clones), which fall within the T6 clade according to the classification of Stothard et al. (*9*). A small number (3/25) of clones showed closest similarity to acanthamoebae belonging to the T4 clade, which includes strains considered to be human pathogens, including some *A. polyphaga* strains.

Acanthamoebae were isolated from fully digested sewage sludge by inoculating diluted sludge onto cerophyl-Prescott infusion agar and subculturing onto nonnutrient agar plates streaked with heat-killed *Escherichia coli*. Cultures were incubated at 20°C and 30°C and examined under an Axioskop 2 microscope (Zeiss, Oberkochen, Germany) at 100× magnification; cells of interest were examined at 1,000× magnification. One clonal population of an *Acanthamoeba* sp. isolated at 20°C, which demonstrated typical trophozoite and cyst morphology, contained large numbers of particles either within vacuoles or within the cytoplasm (Figure). Vacuoles were densely packed with particles that appeared to be constantly moving; vacuole size varied from that typical of food vacuoles to large vacuoles that occupied most of the cell volume (expanded online Figure, panels B, D, and G, www.cdc.gov/EID/content/17/6/1127-F.htm). Because the particles were assumed to be bacterial pathogens, efforts were made to produce an axenic culture of the ameba isolate, and 16S rRNA PCR was performed to identify any intracellular bacteria. DNA was extracted by using a phenol chloroform method according to Griffiths et al. (*6*). However, no 16S rRNA PCR products were amplified.

Months later, an image review led to recognition of unusual arrangements of intracellular particles in a lattice-like structure in which each particle was surrounded by 6 others. Measurement of rows of particles, assuming tight packing, gave an average particle size of 620 nm. At this point, we realized that the particles were virus-like

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PsrP, a Protective Pneumococcal Antigen, Is Highly Prevalent in Children with Pneumonia and Is Strongly Associated with Clonal Type▽

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Invasive pneumococcal disease (IPD) is a major health problem worldwide. Due to ongoing serotype replacement, current efforts are focused in an attempt to identify the pneumococcal antigens that could be used in a next-generation multivalent protein vaccine. The objective of our study was to use real-time PCR to determine the distribution and clonal type variability of PsrP, a protective pneumococcal antigen, among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers. *psrP* was detected in 52.4% of the 441 strains tested. While no differences were determined when the prevalence of *psrP* in colonizing strains ($n = 89$) versus that in all invasive strains ($n = 352$) was compared, a strong trend was observed when the prevalence of *psrP* in all pneumonia isolates ($n = 209$) and colonizing isolates ($P = 0.067$) was compared, and a significant difference was observed when the prevalence in all pneumonia isolates and those causing bacteremia ($n = 76$) was compared ($P = 0.001$). An age-dependent distribution of *psrP* was also observed, with the incidence of *psrP* being the greatest in strains isolated from children >2 years of age ($P = 0.02$). Strikingly, the presence of *psrP* within a serotype was highly dependent on the clonotype, with all isolates of invasive clones such as clonal complex 306 carrying *psrP* ($n = 88$), whereas for sequence type 304, only 1 of 19 isolates carried *psrP*; moreover, this was inversely correlated with antibiotic susceptibility. This finding suggests that inclusion of *psrP* in a vaccine formulation would not target resistant strains. We conclude that *psrP* is highly prevalent in strains that cause IPD but is most prevalent in strains isolated from older children with pneumonia. These data support the potential use of PsrP as one component in a multivalent protein-based vaccine.

Invasive pneumococcal disease (IPD), defined herein as the isolation of *Streptococcus pneumoniae* from normally sterile sites during a clinical syndrome of infection such as bacteremia/sepsis, pneumonia, or meningitis, is an important health problem worldwide. In the year 2000, it is estimated that there were 11 million to 18 million episodes/cases of IPD and 0.7 million to 1 million deaths in children younger than 5 years of age as a result (17). *Streptococcus pneumoniae* is a Gram-positive commensal that colonizes the nasopharynx of healthy children and, less frequently, adults. From the upper respiratory tract, the bacteria can be aspirated into the lungs and can translocate through mucosal cell barriers to the bloodstream and lead to development of IPD (18). This primarily occurs in young children, elderly individuals, and those who are immunocompromised.

The ability of *S. pneumoniae* to cause IPD is dependent on the presence of a polysaccharide capsule that prevents phagocytosis (1). At least 92 chemically and immunologically distinct capsular types (i.e., serotypes) can be produced by the pneumococcus, with certain serotypes more frequently being associated with invasive disease (23). Importantly, while the capsule is requisite for IPD, it is insufficient alone to confer virulence; and an assortment of additional determinants such as adhesins, proteases, toxins, transport systems, and enzymes that modify the extracellular milieu are also required (25). This requirement for noncapsular virulence determinants is proven by human epidemiological studies that show that invasive and noninvasive clonotypes exist within the most invasive serotypes, comparative genomic analyses that find an unequal distribution of noncapsular genes between invasive and noninvasive isolates within the same serotype, and scores of studies that show that deletion of noncapsular genes impact pneumococcal virulence in animal models of pneumonia, sepsis, and meningitis (7, 11, 19, 22).

One recently identified pneumococcal virulence determinant is the pneumococcal serine-rich repeat protein (PsrP), a lung cell and intraspecies bacterial adhesin that is encoded within the 37-kb pathogenicity island called *psrP-secY2A2* (16). PsrP is an extremely large glycosylated cell surface protein that belongs to the serine-rich repeat protein (SRRP) family of Gram-positive bacteria (22). For the pneumococcus, the presence of PsrP has been positively correlated with strains that cause human disease, and PsrP has been shown to mediate adhesion to keratin 10 on lung cells and to mediate the formation of bacterial aggregates in the nasopharynges and lungs of infected mice (21, 22). Antibodies against PsrP neutralize

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bacterial adhesion to cells *in vitro* and inhibit biofilm formation (20, 21). Furthermore, passive immunization of mice with PsrP antiserum or active immunization with recombinant protein protected mice against pneumococcal challenge (20). Thus, PsrP is an important virulence factor by which *S. pneumoniae* is able to cause IPD and is potentially a vaccine candidate.

At this time, considerable resources are being spent in an attempt to identify the pneumococcal antigens that would be used in a next-generation multivalent protein vaccine designed against the pneumococcus. The advantage of such a vaccine is that it would have a lower cost and potentially expanded global coverage compared with the cost and coverage of existing conjugate vaccines. It is generally accepted that multiple antigens will be necessary due to the fact that not all protein determinants are conserved or found within all pneumococcal strains and on their own are not able to confer sufficient protection. To this end, knowledge of the real prevalence of a protein in different clones and serotypes of *Streptococcus pneumoniae* is necessary to consider any protein as a candidate vaccine antigen. Therefore, the objective of our study was to determine the distribution and clonal type variability of PsrP among pneumococcal isolates from children with IPD or healthy nasopharyngeal carriers.

MATERIALS AND METHODS

Strain collection. We analyzed all invasive pneumococcal isolates collected at the Molecular Microbiology Department of the University Hospital Sant Joan de Deu, Barcelona, Spain, from January 2004 to November 2009 ($n = 358$). We have also included 89 strains isolated from the nasopharynges of healthy children during 2004 and 2005 ($n = 89$). A detailed description of our institution and the geographic area was reported elsewhere (13). IPD was defined as the presence of clinical findings of infection, including pneumonia, together with isolation of *S. pneumoniae* in blood, cerebrospinal fluid, or any other normally sterile fluid. The clinical syndrome was classified according to the International Classification of Disease, ninth revision (ICD-9), specific for diseases caused by *S. pneumoniae*, including sepsis, occult bacteremia, meningitis, pneumonia, parapneumonic empyema, peritonitis, arthritis, and endophthalmitis.

Serotyping and antimicrobial susceptibility. All isolates were serotyped by the Quellung reaction at the National Pneumococcus Reference Centre (Majadahonda, Madrid, Spain). In addition, all isolates identified during 2009 were also tested in our laboratory with a rapid specific real-time PCR of the main invasive serotypes according to the methods described for a published assay (24). The agar dilution technique was used to determine the MICs of penicillin and other antibiotics; antibiotic susceptibility was defined according to the 2008 meningeal breakpoints of the Clinical and Laboratory Standards Institute (formerly NCCLS) (14). Isolates with intermediate or high-level resistance were defined as nonsusceptible.

MLST. Genetic characterization was performed using multilocus sequence typing (MLST). In brief, internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl* genes were amplified by PCR from chromosomal DNA of pneumococci using the primer pairs described by Enright and Spratt (5). The sequences of both DNA strands were obtained by use of an ABI 3730xl DNA analyzer (Applied Biosystems). The sequences at each of the seven loci were then compared with the sequences of all of the known alleles at that locus. Sequences that are identical to a known allele were assigned the same allele number, whereas those that differ from any known allele were assigned new allele numbers. The assignment of alleles at each locus was carried out using the software at the pneumococcal web page (www.mlst.net). The alleles at each of the seven loci define the allelic profile of each isolate and their sequence type (ST). Allelic profiles are shown as the combination of 7 alleles in the order *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*. A clone is defined as a group of isolates with identical allelic profiles or STs.

CC. Isolates with genotypes with allelic profiles that differ at only one of the seven loci were called single-locus variants (SLVs). SLVs are sufficiently related to be considered members of a cluster of closely related genotypes, referred to as a clonal complex (CC). Analysis of sequence types and assignment to a clonal complex was performed with the eBURST (based upon related sequence types) program. This program compares a data set of sequence types and groups them into related genotypes and clonal complexes (6).

Extraction of DNA. Genomic DNA was extracted using Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA). Four to 5 CFU/ml was suspended in 100 μl of phosphate-buffered saline (PBS) buffer; 50 μl was transferred to a new microcentrifuge tube and vigorously vortexed with 150 μl of 20% (wt/vol) Chelex-100 in PBS. The bacterium/resin suspensions were incubated for 20 min at 56°C, followed by a 10-min incubation at 99°C. After cooling and centrifugation of the suspensions, the supernatant was used as a template in real-time PCR experiments. Free water and genomic DNA from the *psrP*-carrying TIGR4 and an isogenic mutant deficient in *psrP* (T4 Δ*psrP*) were used as positive and negative controls, respectively (16).

Real-time PCR assay. We analyzed the nucleotide sequence of *psrP* in TIGR4 and all other publically available *S. pneumoniae* genomes available through the United States National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) for primers and probe design. The primers and probe selected were as follows: the forward primer was 5′-CTTTACATTTACCCCTTACGCTGCTA, the reverse primer was 3′-CTGAGAGTGACTTAGACTGTGAAAGTG, and the probe was FAM-CTGGTCGTGCTAGATTC (where FAM is 6-carboxyfluorescein; the quencher was the minor groove binder [MGB] moiety). These primers identified a conserved region within the basic region domain of *psrP* (16).

The reaction volume of 25 μl contained 5 μl of DNA extract from samples or controls and 12.5 μl 2× TaqMan universal master mix (Applied Biosystems), which includes dUTP and uracil-*N*-glycosylase; each primer was used at a final concentration of 300 nM. The TaqMan probe was used at a final concentration of 150 nM. Amplification was done under universal amplification conditions: incubation for 2 min at 50°C (uracil-*N*-glycosylase digestion), 10 min denaturation at 95°C, and 45 cycles of a two-step amplification (15 s at 95°C, 60 s at 60°C). Amplification data were analyzed by SDS software (Applied Biosystems). The reporter dye signal was measured relative to the internal reference dye (carboxy-X-rhodamine) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. The cycle threshold (C_T) value was defined as the cycle at which the reporting dye fluorescence first exceeds the calculated background level.

Statistical analysis. Statistical analysis was performed with the PASW software package (version 17.0). Continuous variables were compared using the *t* test (for approximately normally distributed data) or the Mann-Whitney U test (for skewed data) and were described as mean values and standard deviations or medians and interquartile ranges (IQRs; 25 to 75th percentiles), according to the presence of a normal distribution. The chi-square test or Fisher's exact test (two-tailed) was used to compare categorical variables. Comparison between groups was performed by the Kruskal-Wallis test. Statistical significance was set at a P value of <0.05.

RESULTS

Prevalence of *psrP* in clinical isolates from healthy carriers and individuals with IPD. Of the 358 invasive pneumococcal isolates in our library, 6 of them were not viable and were therefore excluded from the study. As such, we studied a total of 352 invasive pneumococcal isolates and 89 nasopharyngeal pneumococcal isolates (total of 441 strains).

Table 1 shows the prevalence of *psrP* in pneumococcal isolates according to clinical diagnosis, serotype, and clonal type. Overall, we detected *psrP* in 231 (52.4%) of pneumococcal strains tested. No significant differences were found when the prevalence of *psrP* in colonizing strains (43 of 89 isolates; 48.3%) was compared with that in all invasive strains (188 of 352 isolates; 53.4%) ($P = 0.4$). However, a strong trend was observed when the prevalence of *psrP* in all pneumonia isolates (125 of 209; 59.8%) was compared with that in colonizing isolates ($P = 0.067$). The lowest prevalence of *psrP* was found in strains isolated from children with bacteremia (29 of 76 strains; 38.2%). In a breakdown of those causing pneumonia, *psrP* was detected in 62 of 104 (59.6%) isolates causing uncomplicated pneumonia (versus colonizing strains, $P = 0.1$) and 63 of 105 (60%) of isolates from individuals with para-

TABLE 1. Prevalence of positive *psrP* in pneumococcal isolates in children with IPD and healthy carriers by clinical diagnosis, serotype, and clonal type

Subject group and clinical diagnosis	No. of strains *psrP* positive/total no. of strains (%)	Serotype		Clonal type	
		Main serotype	No. of strains *psrP* positive/total no. of strains (%)	Main clonal type	No. of strains *psrP* positive/total no. of strains (%)
IPD patients	188/352 (53.4)	1	84/104 (80.8)	CC306	82/82 (100)
Pneumonia	125/209 (59.8)			ST304	1/18 (5.5)
		19A	15/36 (41.7)	CC230	2/9 (22.2)
				ST2013	2/7 (28.5)
				ST1201	5/5 (100)
		5	15/20 (75)	CC289	15/20 (75)
		7F	2/13 (15.3)	ST191	2/12 (16.6)
		Others[a]	9/36 (25)		
Bacteremia	29/76 (38.2)	19A	12/20 (60)	ST1201	7/7 (100)
				ST320	0/4 (0)
		7F	0/9 (0)	ST191	0/9 (0)
		14	0/5 (0)	CC156	0/2 (0)
		19F	3/5 (60)	CC177	2/2 (100)
		Others[b]	14/37 (37.8)		
Meningitis	25/49 (51)	19A	5/8 (62.5)	CC199	2/2 (100)
				CC230	0/2 (0)
		19F	4/5 (80)	CC177	2/2 (100)
		6A	2/4 (50)	ST1692	1/2 (50)
		7F	1/3 (33.3)	ST191	1/3 (33.3)
		Others[c]	13/29 (44.8)		
Other clinical syndromes[d]	9/18 (50)				
		19A	2/4 (50)	CC230	0/1 (0)
				ST320	0/1 (0)
		1	2/2 (100)	CC306	2/2 (100)
		19F	1/2 (50)	ST2948	1/1 (100)
		23F	0/2 (0)	ST109	0/1 (0)
		Others[e]	4/8 (50)		
Healthy carriers	43/89 (48.3)	19A	6/9 (66.6)	CC199	2/2 (100)
				CC202	1/2 (50)
		6A	6/9 (66.6)	CC97	1/1 (100)
				ST1143	1/1 (100)
		19F	5/7 (71.4)	CC177	2/2 (100)
		15B	4/6 (66.7)	ST101	1/2 (50)
		23B	1/6 (16.7)	ST2372	0/1 (0)
		6B	2/5 (40)	ST386	1/1 (100)
		9V	1/5 (20)	CC156	0/1 (0)
		Others[f]	18/42 (42.8)		

[a] Other serotypes in pneumonia: S14 ($n = 9$); S3 ($n = 6$); S24F/B ($n = 6$); S6A ($n = 3$); S6B ($n = 3$); S9V ($n = 3$); and S10A, S15B, S18C, S2, S38, and S4 ($n = 1$ each).

[b] Other serotypes in bacteremia: S1 ($n = 4$); S3 ($n = 4$); S5 ($n = 4$); 23B ($n = 3$); S38 ($n = 3$); S6A ($n = 3$); S10A ($n = 2$); S15B ($n = 2$); S23F ($n = 2$); S34 ($n = 2$); and S12F, S18C, S21, S22F, S24F, S27, S35B, and S4 ($n = 1$ each).

[c] Other serotypes in meningitis: S15C, S18C, S22, S23B, S23F, S3, S5, S6BS, and 24F ($n = 2$ each) and S1, S10A, S12F, S13, S14, S15A, S16, S16F, S27, S31, and S9N ($n = 1$ each).

[d] Other clinical syndromes: arthritis ($n = 11$), appendicitis ($n = 5$), pericarditis ($n = 1$), and peritonitis ($n = 1$).

[e] Other serotypes in other clinical syndromes: S14, S28, S35B, S38, S4, S5, S6A, and S7F ($n = 1$ each).

[f] Other serotypes in healthy carriers: S21 ($n = 4$); S23F ($n = 4$); S10A ($n = 3$); S15A ($n = 3$); S23A ($n = 3$); S3 ($n = 3$); S1 ($n = 2$); S15C ($n = 2$); S24 ($n = 2$); S29 ($n = 2$); S35B ($n = 2$); S37 ($n = 2$); and S10, S11, S16, S17, S28, S31, S38, S37, 7F, and 9N ($n = 1$ each).

pneumonic empyema (versus colonizing strains, $P = 0.19$). Thus, consistent with its role as a lung cell adhesin, *psrP* was more frequently present in pneumonia isolates than in colonizing isolates, albeit not to a significant level, and was present at a significantly higher rate in pneumonia isolates than in those causing bacteremia ($P = 0.001$). Surprisingly, we also observed an age-dependent distribution for *psrP* in clinical isolates from children with IPD. *psrP* was detected in 76 of 175 (46.1%) strains isolated from children with IPD less than 24 months old, while in older children, this rate was significantly higher: 112 of 187 (59.9%) ($P = 0.01$).

Specifically, the prevalence of *psrP* in pneumonia isolates from children less than 24 months was 49.3% (36 of 73 strains), and that in isolates from older children was 65.4% (89 of 136 strains) ($P = 0.02$). Moreover, for isolates from older children, this 65.4% prevalence of *psrP* was significantly higher than that for nonpneumonia IPD isolates (56 of 116; 48.3%; $P = 0.006$) and that for colonizing isolates (33 of 65; 50.8%; $P = 0.04$).

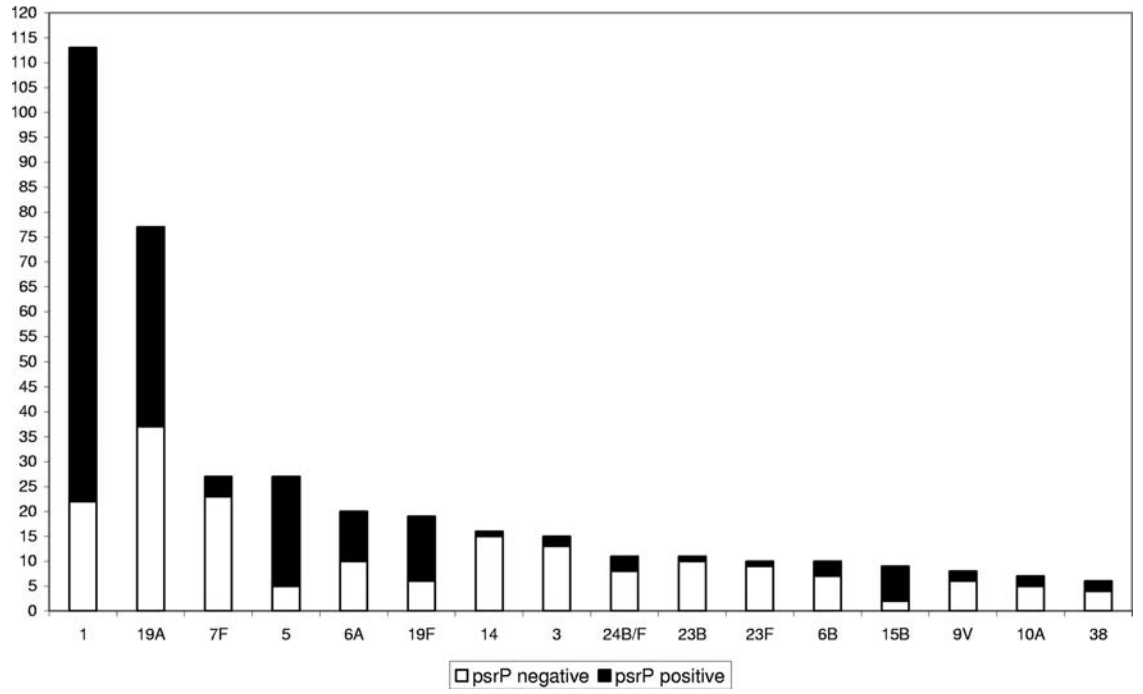

FIG. 1. Prevalence of *psrP* according to serotype of *Streptococcus pneumoniae* in groups of more than five isolates.

Prevalence of *psrP* according to serotype of isolates. A total of 37 different serotypes were detected among the isolates causing IPD and 29 were detected among those from carriers. Significant differences in the prevalence of *psrP* were observed according to the serotype of the strains ($P < 0.0001$). Figure 1 show the distribution of *psrP* according to serotypes with more than 5 isolates. *psrP* was observed at very high frequencies in some epidemic serotypes, such as serotype 1 (91 of 113 isolates; 80.5%) and serotype 5 (22 of 27 isolates; 81.5%). In contrast, positivity for *psrP* was rarely detected in other serotypes, such as serotype 3 (2 of 15 isolates; 13.3%), serotype 7F (4 of 27 isolates; 14.8%), and serotype 14 (1 of 16 strains; 6.3%). The prevalence of *psrP* in serotype 19A, which is an emergent serotype in the geographic area where the isolates for this study were collected (12), was 51.9% (40 of 77 strains).

Importantly, the prevalence of *psrP* in serotypes included in the 7-valent conjugate vaccine (PCV-7; 26 of 70 strains; 37.1%) was significantly lower than that in nonvaccine serotypes (205 of 371; 55.3%) ($P = 0.006$). In contrast, in the context of the newly approved 10-valent pneumococcal conjugate vaccine (PCV-10) and 13-valent pneumococcal conjugate vaccine (PCV-13), the difference between vaccine and nonvaccine strains was switched. For PCV-10, a higher rate of positivity for *psrP* in the vaccine serotypes was observed: 143 of 237 (60.3%) among PCV-10 serotypes versus 88 of 204 (43.1%) among non-PCV-10 serotypes ($P < 0.0001$). For PCV-13, *psrP* was detected in 195 of 349 (55.9%) strains of PCV-13 serotypes and 36 of 92 (39.1%) strains of non-PCV-13 serotypes ($P = 0.004$).

Prevalence of *psrP* according to antibiotic susceptibility of strains. Antibiotic susceptibility information was obtained for 432 of 441 total isolates (98%). In general, a positive correlation between the presence of *psrP* and antibiotic susceptibility was observed. The prevalence of *psrP* in penicillin-susceptible strains from children with IPD was 64.5% (171 of 265 strains), whereas it was only 15.2% (12 of 79 strains) in nonsusceptible strains ($P < 0.0001$). The same pattern was observed for cefotaxime, tetracycline, and erythromycin (Table 2). Strikingly, *psrP* was absent in almost all strains with high-level resistance to penicillin or cefotaxime: only 2 (10%) of 20 strains with penicillin MICs of ≥ 2 μg/ml and none of 6 strains with cefotaxime MICs of ≥ 2 μg/ml were positive for *psrP*. No significant differences in *psrP* prevalence were found according to the susceptibilities of nasopharyngeal strains in healthy carriers.

Prevalence of *psrP* according to clonal type. Sequence and clonal type analyses were performed for 372 of 441 strains (84% of the collection). A total of 94 different sequence types were found, and these were grouped into 17 clonal complexes and 52 SLVs. The overall prevalence of *psrP* in this collection was 54.8% (204 of 372).

A significant difference in the prevalence of *psrP* was observed according to clonal type ($P < 0.0001$). Moreover, the presence or absence of *psrP* was closely related to specific genotypes but not to specific serotypes. Figure 2 shows the relative frequency of *psrP* among genotypes with more than 5 isolates. In brief, *psrP* was present in all 89 isolates (100%) belonging to CC306 (all of them serotype 1) and, in contrast, was absent in 18 of 19 isolates (5.3%) of serotype 1 belonging to ST304. Similarly, all 15 isolates belonging to ST1201 and all 11 strains belonging to CC199 (all of them serotype 19A) were positive for *psrP*. In contrast, 100% of 9 strains of serotype 19A belonging to multiresistant clone ST320 and 87% of 23 strains belonging to multiresistant clone CC230 (16 serotype 19A isolates and 7 serogroup 24 isolates) were negative for *psrP*. Other clones with a high prevalence of *psrP* were CC177 (7 of 7 isolates) and CC289 (21 of 26 isolates).

TABLE 2. Prevalence of positivity for *psrP* according to antimicrobial resistance (meningeal breakpoints) in pneumococcal isolates in children with IPD and healthy carriers

Subject group, antimicrobial, and MIC	No. of strains *psrP* positive/total no. of strains (%)	*P*
Patients with IPD		
Penicillin		
MIC ≤ 0.06 μg/ml	171/265 (64.5)	<0.000
MIC ≥ 0.12 μg/ml	12/79 (15.2)	
Cefotaxime		
MIC ≤ 0.5 μg/ml	180/307 (58.6)	<0.000
MIC = 1 μg/ml	3/31 (9.7)	
MIC ≥ 2 μg/ml	0/6 (0)	
Erythromycin		
MIC ≤ 0.25 μg/ml	162/267 (60.7)	<0.000
MIC = 0.5 μg/ml	0/0	
MIC ≥ 1 μg/ml	21/77 (27.3)	
Tetracycline		
MIC ≤ 2 μg/ml	160/262 (61.1)	<0.000
MIC = 4 μg/ml	1/1	
MIC ≥ 8 μg/ml	21/78 (26.9)	
Healthy carriers		
Penicillin		
MIC ≤ 0.06 μg/ml	33/61 (54.1)	0.10
MIC ≥ 0.12 μg/ml	9/27 (33.3)	
Cefotaxime		
MIC ≤ 0.5 μg/ml	38/76 (50)	0.49
MIC = 1 μg/ml	3/10 (30)	
MIC ≥ 2 μg/ml	1/2 (50)	
Erythromycin		
MIC ≤ 0.25 μg/ml	30/62 (48.4)	1
MIC = 0.5 μg/ml	0/0	
MIC ≥ 1 μg/ml	12/26 (46.2)	
Tetracycline		
MIC ≤ 2 μg/ml	34/65 (52.3)	0.22
MIC = 4 μg/ml	0/0	
MIC ≥ 8 μg/ml	8/23 (34.8)	

DISCUSSION

The current conjugate pneumococcal vaccines, in which capsular polysaccharides are bound to either diphtheria or tetanus toxoid, are immunogenic and efficacious in children and prevent disease caused by the serotypes whose capsule types are in the vaccine (2, 4, 26). However, as these vaccines do not cover the full spectrum of invasive pneumococcal serotypes, temporal and geographic changes in serotype frequency associated with IPD exist. The pneumococcus is able to replace its capsule type through natural transformation, and children remain at risk of infection by nonvaccine serotypes. Moreover, the possibility of serotype shift, where the nonvaccine serotypes acquire an ecological advantage for increased carriage prevalence and, concomitantly, disease, remains real (8).

A possible solution to this problem is either replacement of the carrier toxoid with a conserved and highly antigenic single pneumococcal protein, thus providing serotype-independent protection, or alternatively, if a single antigen is insufficient, creation of a multivalent protein vaccine that eschews the capsular polysaccharide entirely. At this time, it is not clear which approach is best or which pneumococcal protein(s) should be included in any revised vaccine formulations. To address these issues, detailed molecular epidemiology is required to assess the frequency and distribution of various pneumococcal determinants in invasive clinical isolates. This is the first large study analyzing the prevalence of *psrP* in pneumococcal isolates from children with IPD and healthy nasopharyngeal carriers. The results of our study are in agreement with published data regarding the function of this new pneumococcal virulence factor and provide clues to the forces responsible for the spread of the pathogenicity island encoding PsrP, *psrP-secY2A2*, among different serotypes and clones.

The increased frequency of the gene encoding PsrP in clinical isolates from individuals with pneumonia compared with the frequency in those isolated from the nasopharynges of healthy carriers or children with bacteremia is consistent with published findings showing that PsrP is exclusively a lung cell adhesin and that it does not play a role in the nasopharynx during colonization or in the bloodstream in an intraperitoneal model of sepsis. These data also suggest that PsrP alone would protect against only 60% of strains that are capable of causing pneumonia. Thus, these findings indicate that, at best, PsrP could be a single component of a multicomponent vaccine formulation. The inclusion of a second or third protein that protects against bacteremia and whose coverage helps to cover the ~40% of invasive isolates that lack PsrP would be required.

Given that *psrP* was found to be predominantly associated with antimicrobial-susceptible isolates, it can be inferred that its inclusion within any vaccine would not serve as a mechanism to decrease the incidence of existing multidrug-resistant pneumococcal isolates. It also suggests that the extensive use of antimicrobials within the community is not responsible for promoting the preponderance of clonotypes that carry *psrP*. Counter to the latter view, we have recently shown that PsrP mediates the formation of bacterial aggregates within the lungs and the formation of more dense biofilms *in vitro* (20). As *psrP* is predominantly found in antimicrobial-susceptible isolates and bacterial aggregates and biofilms are considered to be more resistant to antimicrobials, it is a distinct possibility that in the absence of a dedicated antimicrobial resistance mechanism, PsrP confers resistance to susceptible isolates *in vivo* through enhanced biofilm formation. Thus, antimicrobial pressures may be serving to maintain *psrP* within susceptible clonotypes. To test this hypothesis, ongoing experiments are testing the resistance of these PsrP-mediated aggregates to antimicrobials.

When we stratified the incidence of *psrP* in children with pneumonia by age, we found that 65.4% of pneumococcal strains that caused disease in children greater than 2 years of age carry this virulence gene. This rate was significantly higher that the rate found in strains that cause disease in young children. One possible explanation for this may be the serotype distribution of *psrP*. *psrP* was found to be predominant in serotypes not covered by the 7-valent conjugate vaccine. In the prevaccine era, the 7 serotypes included in the vaccine were most prominent in Europe. Thus, the discrepancy in age might be explained by the fact that the infant nasopharynx is first

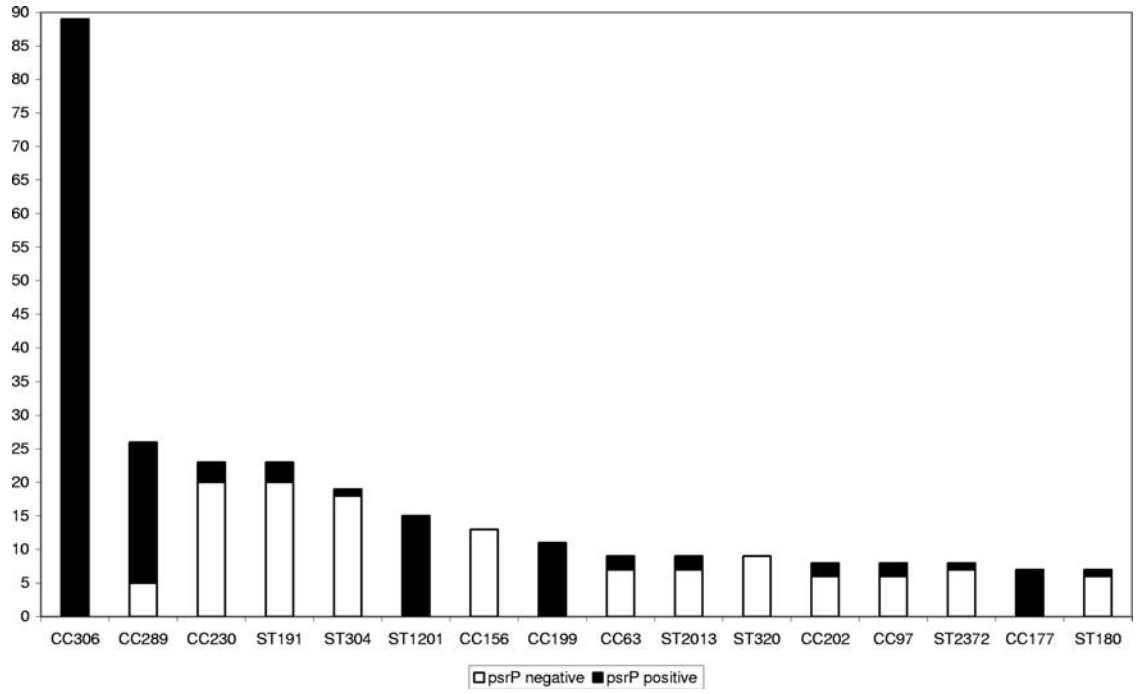

FIG. 2. Prevalence of *psrP* according to clonal type of *Streptococcus pneumoniae* in groups of more than five isolates.

occupied by these 7 serotypes and is then filled with those from the nonvaccine serotypes at a later age. Studies by Melegaro and colleagues would support this explanation (10). However, in our study, this is not a valid explanation because PCV-7 serotypes were not found to be predominant in either younger children or older children (36 of 189 [19%] in children less than 2 years of age versus 34 of 252 [13.5%] in children ≥2 years of age; $P = 0.1$). Thus, other reasons, which are not yet clear, must explain why strains that carry *psrP* cause IPD at a later age than those that do not. Importantly, the fact that *psrP* is found predominantly in the PCV-7 nonvaccine serotypes suggests that its inclusion would expand coverage beyond that of the current vaccine. However, this is less so for the PCV-10 and PCV-13 formulations due to the inclusion of serotypes 1 and 19A, the strains of which have a high frequency of *psrP*. Of note, the reported prevalence of *psrP* among PCV-7 serotypes may be skewed by the fact that relatively few PCV-7 serotype strains were isolated in our study. It would therefore be interesting to test a collection of clinical isolates archived prior to the introduction of the vaccine. Such a study would determine if the current 60% incidence of *psrP* in pneumonia clinical isolates was due to serotype replacement (i.e., positive selection of nonvaccine serotypes with clones that carry *psrP*) or if *psrP* has been prevalent all along among serotypes and clones that frequently cause pneumonia.

Finally, we observed the presence of *psrP* in certain clonotypes and its absence in others. For example, all strains of serotype 1 with CC306 were positive for *psrP*, while only 1 of 18 strains with ST304 was positive. ST306, together with ST304, ST228, and others, belongs to lineage A of serotype 1, which is the major lineage detected in North America and Europe (3). According to some epidemiological studies ST306 has been related to several outbreaks of invasive pneumococcal disease (9) and the emergence of pleuropneumonia in the vaccine era (15), while ST304 has not. The high prevalence of PsrP, a lung cell bacterial adhesin, in ST306 strains could be associated with this fact. It is not clear why *psrP* would be present in some isolates but not others; however, this suggests that other pneumococcal virulence determinants compensate for the absence of PsrP. Thus, detailed comparative genomic analyses of invasive clonotypes within the same serotype containing *psrP* and not containing *psrP* may be warranted to identify the compensatory determinants that are responsible for disease and, moreover, to determine if their inclusion along with PsrP in a multicomponent vaccine would enhance coverage.

In conclusion, *psrP* is highly prevalent in our clinical collection and is mainly present in strains isolated from older children with pneumonia. The distribution of *psrP* seemed to be strongly associated with antimicrobial sensitivity, non-PCV-7 serotypes, and specific clonotypes of pneumococci. These data support the potential use of PsrP as a protective antigen in the design of a next generation of protein-based combination vaccines. However, the data also indicate that additional components that fill the bacteremia and serotype niche not covered by PsrP are required.

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REFERENCES

1. **Alonso de Velasco, E., A. F. Verheul, J. Verhoef, and H. Snippe.** 1995. Streptococcus pneumoniae: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. **59:**591–603.
2. **Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohberger, W. Watson, R. Austrian, and K. Edwards.** 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal vaccine in children. Pediatr. Infect. Dis. J. **19:**187–195.
3. **Brueggemann, A. B., and B. G. Spratt.** 2003. Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates. J. Clin. Microbiol. **41:**4966–4970.
4. **Cutts, F. T, S. M. Zaman, G. Enwere, S. Jaffar, O. S. Levine, J. B. Okoko, C. Oluwalana, A. Vaughan, S. K. Obaro, A. Leach, K. P. McAdam, E. Biney, M. Saaka, U. Onwuchekwa, F. Yallop, N. F. Pierce, B. M Greenwood, R. A. Adegbola, and Gambian Pneumococcal Vaccine Trial Group.** 2005. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet **365:**1139–1146.
5. **Enright, M. C., and B. G. Spratt.** 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology **144:**3049–3060.
6. **Feil, E. J., B. C. Li, D. M. Aanensen, W. P. Hanage, and B. G. Spratt.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J. Bacteriol. **186:**1518–1530.
7. **Glover, D. T., S. K. Hollingshead, and D. E. Briles.** 2008. *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect. Immun. **76:**2767–2776.
8. **Hanage, W. P., C. Fraser, J. Tang, T. R. Connor, and J. Corander.** 2009. Hyper-recombination, diversity, and antibiotic resistance in pneumococcus. Science **324:**1454–1457.
9. **Henriques-Normark, B., M. Kalin, A. Ortqvist, T. Akerlund, B. O. Liljequist, J. Hedlund, S. B. Svenson, J. Zhou, B. G. Spratt, S. Normark, and G. Källenius.** 2001. Dynamics of penicillin-susceptible clones in invasive pneumococcal disease. J. Infect. Dis. **184:**861–869.
10. **Hussain, M., A. Melegaro, R. G. Pebody, R. George, W. J. Edmunds, R. Talukdar, S. A. Martin, A. Efstratiou, and E. Miller.** 2005. A longitudinal household study of Streptococcus pneumoniae nasopharyngeal carriage in a UK setting. Epidemiol. Infect. **133:**891–898.
11. **Kadioglu, A., J. N. Weiser, J. C. Paton, and P. W. Andrew.** 2008. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat. Rev. Microbiol. **6:**288–301.
12. **Muñoz-Almagro, C., C. Esteva, M. F. de Sevilla, L. Selva, A. Gene, and R. Pallares.** 2009. Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. J. Infect. **59:**75–82.
13. **Muñoz-Almagro, C., I. Jordan, A. Gene, J. J. Garcia-Garcia, and R. Pallares.** 2008. Emergence of invasive pneumococcal disease caused by non-vaccine serotypes in the era of 7-valent conjugate vaccine. Clin. Infect. Dis. **46:**174–182.
14. **NCCLS/CLSI.** 2008. Performance standards for antimicrobial susceptibility testing. Eighteenth informational supplement. CLSI document M100-S18. Clinical and Laboratory Standards Institute, Wayne, PA.
15. **Obando, I., C. Muñoz-Almagro, L. A. Arroyo, D. Tarrago, D. Sanchez-Tatay, D. Moreno-Perez, S. S. Dhillon, C. Esteva, S. Hernandez-Bou, J. J. Garcia-Garcia, W. P. Hausdorff, and A. B. Brueggemann.** 2008. Pediatric parapneumonic empyema, Spain. Emerg. Infect. Dis. **14:**1390–1397.
16. **Obert, C., J. Sublett, D. Kaushal, E. Hinojosa, T. Barton, E. I. Tuomanen, and C. J. Orihuela.** 2006. Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. Infect. Immun. **74:**4766–4777.
17. **O'Brien, K. L., L. J. Wolfson, J. P. Watt, E. Henkle, M. Deloria-Knoll, N. McCall, E. Lee, K. Mulholland, O. S. Levine, T. Cherian, and Hib and Pneumococcal Global Burden of Disease Study Team.** 2009. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet **374:**893–902.
18. **Orihuela, C. J., and E. I. Tuonamen.** 2009. Models of pneumococcal disease. Drug Discov. Today Dis. Models **3:**69–75.
19. **Rajam, G., J. M. Anderton, G. M. Carlone, J. S. Sampson, and E. W. Ades.** 2008. Pneumococcal surface adhesin A (PsaA): a review. Crit. Rev. Microbiol. **34:**131–142.
20. **Rose, L., P. Shivshankar, E. Hinojosa, A. Rodriguez, C. J. Sanchez, and C. J. Orihuela.** 2008. Antibodies against PsrP, a novel *Streptococcus pneumoniae* adhesin, block adhesion and protect mice against pneumococcal challenge. J. Infect. Dis. **198:**375–383.
21. **Sanchez, C. J., P. Shivshankar, K. Stol, S. Trakhtenbroit, P. M. Sullam, K. Sauer, P. W. M. Hermans, and C. J. Orihuela.** 2010. The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation *in vivo* and in biofilms. PloS Pathog. **6:**e1001044.
22. **Shivshankar, P., C. Sanchez, L. F. Rose, and C. J. Orihuela.** 2009. The *Streptococcus pneumoniae* adhesin PsrP binds to keratin 10 on lung cells. Mol. Microbiol. **73:**663–679.
23. **Sniadack, D. H., B. Schwartz, H. Lipman, J. Bogaert, J. C. Butler, R. Dagan, G. Echaniz-Aviles, N. Lloyd-Evans, A. Fenoll, N. I. Girgis, J. Henrichsen, K. Klugman, D. Lehmann, A. K. Takala, J. Vandepitte, S. Gove, and R. F. Breiman.** 1995. Potential interventions for the prevention of childhood pneumonia: geographic and temporal differences in serotype and serogroup distribution of sterile site pneumococcal isolates from children—implications for vaccine strategies. Pediatr. Infect. Dis. J. **14:**503–510.
24. **Tarrago, D., A. Fenoll, D. Sanchez-Tatay, L. A. Arroyo, C. Muñoz-Almagro, C. Esteva, W. P. Hausdorff, J. Casal, and I. Obando.** 2008. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin. Microbiol. Infect. **14:**828–834.
25. **Van der Poll, T., and S. M. Opal.** 2009. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet **374:**1543–1556.
26. **Whitney, C. G., T. Pilishvili, M. M. Farley, W. Schaffner, A. S. Craig, R. Lynfield, A. C. Nyquist, K. A. Gershman, M. Vazquez, N. M. Bennett, A. Reingold, A. Thomas, M. P. Glode, E. R. Zell, J. H. Jorgensen, B. Beall, and A. Schuchat, A.** 2006. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet **368:**1495–1502.

Title: Prevalence and clonal distribution of *pcpA, psrP* and Pilus-1 among pediatric isolates of *Streptococcus pneumoniae*

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ABSTRACT

Streptococcus pneumoniae is the leading cause of vaccine-preventable deaths globally. The objective of this study was to determine the distribution and clonal type variability of three potential vaccine antigens: Pneumococcal serine-rich repeat protein (PsrP), Pilus-1, and Pneumococcal choline binding protein A (PcpA) among pneumococcal isolates from children with invasive pneumococcal disease and healthy nasopharyngeal carriers. We studied by Real-Time PCR a total of 458 invasive pneumococcal isolates and 89 nasopharyngeal pneumococcal isolates among children (total=547 strains) collected in Barcelona, Spain, from January 2004 to July 2010. *pcpA*, *psrP* and pilus-1 were detected in 92.8%, 51.7% and 14.4% of invasive isolates and in 92.1%, 48.3% and 18% of carrier isolates, respectively. Within individual serotypes the prevalence of *psrP* and pilus-1 was highly dependent on the clonal type. *pcpA* was highly prevalent in all strains with the exception of those belonging to serotype 3 (33.3% in serotype 3 isolates vs. 95.1% in other serotypes; P<.001). *psrP* was significantly more frequent in those serotypes that are less apt to be detected in carriage than in disease; 58.7% vs. 39.1% P<.001. Antibiotic resistance was associated with the presence of pilus-1 and showed a negative correlation with *psrP*. These results indicate that PcpA, and subsequently Psrp and Pilus-1 together might be good candidates to be used in a next-generation of multivalent pneumococcal protein vaccine.

INTRODUCTION

Invasive disease caused by *Streptococcus pneumoniae* is responsible for more than 1.6 million childhood deaths worldwide every year [1]. In certain developed countries, including Spain, despite vaccination with a 7-valent conjugate vaccine against capsular polysaccharide (PCV-7), pneumococcal pneumonia remains a major cause of pediatric hospital admission [2, 3, 4]. PCV-7 is composed of capsular polysaccharide from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F individually conjugated to diphtheria CRM197 and has proved to be effective in preventing pneumococcal disease caused by these serotypes in children [5]. PCV7 also prevents invasive pneumococcal disease (IPD) in adult and non-vaccinated children by an indirect effect (herd immunity) on pneumococcal transmission [5, 6]. Importantly, nowadays evidence exists of the emergence of non-vaccine serotypes in children and adults to occupy this vaccine-emptied niche, thereby partially eroding the benefit of PCV-7 [3, 7, 8, 9]. For example, in Spain disease caused by serotype 19A was responsible for 13.5% of pediatric IPD during the period 2000-2008, whereas in 2000, at the time of introduction of PCV-7, serotype 19A only accounted for 4.6% of pediatric infections [10]. The pneumococcus is also a primary cause of otitis media and PCV-7 only slightly reduces the rate of disease [11]. At present, more than 1,500,000 cases occur annually in the United States, with an estimated cost of 440 million U.S. dollars [12]. Thus, pneumococcal disease remains a major medical problem with an urgent need for an improved vaccine.

Due to these limitations, other conjugate vaccines with a larger number of serotypes have been recently commercialized. These include a 10-valent conjugate vaccine (PCV10), which includes the seven serotypes of PCV7 plus serotypes 1, 5 and 7F and PCV13 (PCV10 plus additional serotypes 3, 6A and 19A). These vaccines will most likely continue to reduce the burden of invasive pneumococcal disease and are becoming increasingly available in underdeveloped countries due to efforts of institutions such as The Bill and Melinda Gates Foundation through GAVI Alliance [13, 14]. However, due to the high cost of the conjugation process, these vaccines are limited in the number of serotypes that can be included in an affordable vaccine. The current cost for each dose of PCV13 is $100-125, with three immunizations recommended.

An alternate vaccine strategy is the use of a serotype-independent vaccine using conserved common pneumococcal protein antigens. These might stand alone, or replace the diphtheria toxoid in the conjugate vaccine and thereby enhance coverage of the existing vaccines. To date, numerous preclinical studies have shown that different pneumococcal proteins confer protection against pneumococcal challenge and that a combination of multiple proteins confers superior protection. The main advantage of a protein vaccine is that protection would not be serotype dependent and fewer antigen candidates could offer a high coverage with a lower cost of manufacturing. For these reasons, studies are warranted in determining if a next-generation of a multivalent protein vaccine against pneumococcus is feasible and desirable. .

The objective of the present study was to determine the distribution and clonal type variability of three novel potential vaccine candidates: Pneumococcal serine-rich protein (PsrP), Pilus-1, and Pneumococcal choline binding protein A (PcpA). PsrP is a serine rich repeat protein (SRRP) previously demonstrated to be responsible for lung-cell attachment and *in vivo* biofilm formation [15, 16]. Pilus is a long organelle that, like PsrP, extends beyond the polysaccharide capsule and acts as an adhesin [17]. Finally, PcpA is a choline-binding protein with a role in pneumococcal adhesion and biofilm formation [18, 19]. Determining the prevalence and distribution of these proteins in strains that cause IPD and their correlation with disease and antibiotic resistance could be of great value for future vaccine formulations.

METHODS

Clinical isolates. All pediatric invasive pneumococcal isolates characterized by the Molecular Microbiology Department at University Hospital Sant Joan de Deu in Barcelona, Spain from January 2004 to December 2010 were included in this study. The department performs molecular surveillance of pneumococci in Catalonia, Spain. Clinical isolates were obtained from patients admitted to Sant Joan de Déu Hospital and, since 2009, from patients attended in 30 health centers throughout Catalonia region. In addition, we also included eighty-nine pneumococcal strains isolated from nasopharynx of healthy children during 2004-2008.

Serotyping and antimicrobial susceptibility. All isolates were serotyped by Quellung reaction at the National Pneumococcus Reference Centre (Majadahonda, Madrid). Pneumococcal isolates collected since 2009 were also serotyped by Real-Time PCR (RT-PCR) using published protocols [20]. Serotypes were classified according to coverage of the existing 7, 10, and 13-valent conjugate vaccines and their attack rate according to the studies of Brueggemann *et al.* [21] and Sleeman *et al.* [22]. Serotypes with high attack rate (those that are less apt to be detected in carriage than in disease) included: 1, 4, 5, 7F, 9V, 14, 18C and 19A. Serotypes with low attack rate (that are less apt to be detected in disease than in carriage) included: 3, 6A, 6B, 8, 9N, 10A, 11A, 12F, 13, 15A, 15BC, 16F, 17F, 19F, 20, 21, 22F, 23A, 23B, 23F, 24F, 27, 31, 33F, 35B, 35F, 37 and 38. Agar dilution technique was used to determine the minimal inhibitory concentrations (MICs) of penicillin and other antibiotics. Antibiotic susceptibility was defined according to the 2008 meningeal breakpoints established by the Clinical Laboratory Standards Institute [23]. Isolates with intermediate or high level resistance were defined as non-susceptible.

Extraction of DNA. Genomic DNA was extracted from bacteria using Chelex-100 resin (BioRad Laboratories, Hercules, California, USA). Briefly, pneumococci scraped from blood agar plates were suspended in 100 µl of PBS-buffer; 50 µl were transferred to a new microcentrifuge tube and vigorously vortexed with 150 µl of 20% w/v Chelex-100 in

PBS. The bacteria/resin suspensions were incubated for 20 minutes at 56ºC followed by a 10-minute incubation at 100ºC. After cooling and centrifugation, the supernatant was used as a DNA template in PCR reactions.

Multilocus Sequence typing (MLST). Genetic characterization of pneumococci was performed using MLST. In brief, internal fragments of the *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt* and *ddl* genes were amplified by PCR using the primer pairs described by Enright and Spratt [24]. PCR products were sequenced using an ABI 3130xl GeneticAnalyzer (Applied Biosystems). The sequences at each of the seven loci were then compared with all of the known alleles at that locus. Sequences that are identical to a known allele were assigned the same allele number whereas those that differ from any known allele were assigned new allele numbers. The assignment of alleles at each locus was carried out using the software at the pneumococcal web page: www.mlst.net. The alleles at each of the seven loci define the allelic profile of each isolate and their sequence type (ST). Allelic profiles are shown as the combination of 7 alleles in the order *aroE, gdh, gki, recP, spi, xpt and ddl*. A clone is defined as a group of isolates with identical allelic profile or ST.

Real-Time PCR assay. We analyzed the nucleotide sequence of *psrP,* pilus-1 subunit *rrgC*, and *pcpA* for primers in all publically available *S. pneumoniae* genomes available through the United States National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).The primers and probe selected for *psrP* detection were: forward primer: 5'-CTTTACATTTACCCCTTACGCTGCTA; reverse primer 3' CTGAGAGTGACTTAGACTGTGAAAGTG and probe: FAM-CTGGTCGTGCTAGATTC (Quencher MGB). These primers identified a conserved region within Basic Region domain of PsrP. For pilus-1 detection the primers and probe were: forward primer: 5'-TTGTGACAAATCTTCCTCTTGGGA; reverse primer: 3'-GTCACCAGCTGATGATCTACCA and probe: FAM-CAGTGGCTCCACCTCC (Quencher MGB). These primers identified a conserved region within the structural subunit protein RrgC encoded in the *rlrA* islet of pilus type 1. For *pcpA* detection the primers and probe were: forward primer: 5'-

GAAAAAGTAGATAATATAAAACAAGAAACTGATGTAGCTAAA; reverse primer: 3'-ACCTTTGTCTTTAACCCAACCAACT and probe: FAM-CTCCCTGATTAGAATTC (Quencher MGB). These primers identified a conserved region of N-terminal fragment of PcpA. Finally, as a positive control and to test PCR inhibitors and DNA quality, detection of *ply* gene by Real-Time PCR was performed as previously described in all strains [25]. *Ply* encodes the pneumolysin, a toxin found within all *S.pneumoniae.*

The reaction volume for each gene detected was a total of 25µl and contained 5µl of DNA extract from samples or controls and 12.5µl 2X TaqMan Universal Master Mix (Applied Biosystems), which includes dUTP and uracil-N-glycosylase; each primer was used at a final concentration of 900nM. The TaqMan probes were used at a final concentration of 250nM. DNA Amplification was done performing universal amplification conditions: incubation for 2 min at 50℃ (uracil-N- glycosylase digestion) and 10 min denaturation at 95℃, 45 cycles of two-step amplifi cation (15 s at 95℃, 60 s at 60℃). Amplification data were analyzed by SDS software (Applied Biosystems). The reporter dye was measured relative to the internal reference dye (ROX) signal to normalize for non-PCR related fluorescence fluctuations occurring from well to well. The cycle threshold (CT) value was defined as the cycle at which the reporting dye fluorescence first exceeds the background level.

Statistical analysis. Statistical analysis was performed with the PASW software package (version 17.0). Continuous variables were compared using the t test (for approximately normally distributed data) or the Mann-Whitney U test (for skewed data) and described as mean values and standard deviations or median and interquartile range P25-P75 (IQR) according to the presence of normal distribution. Chi-square test or Fisher's exact test (two-tailed) was used to compare categorical variables. Comparison between groups was performed by Kruskal-Wallis test. Statistical significance was set at a *P* value of <0.05.

RESULTS

Strain properties. Of the total 461 pediatric invasive pneumococcal isolates in our library, 3 of them could not be recovered from stocks and were thereby excluded from the study. As such, we examined a total of 458 invasive pneumococcal isolates and 89 nasopharyngeal pneumococcal isolates among children (total=547 strains).

The clinical syndromes were: pneumonia 257 (111 of them with empyema), bacteremia 114, meningitis 68, arthritis 13, appendicitis 4, pericarditis 1 and peritonitis 1.

The most frequent serotypes detected among invasive isolates were serotype 1 (n=134), 19A (n=84), 7F (n=35), 5 (n=34) and 14 (n=19). Among carriers the most frequent serotypes were 19A (n=9), 6A (n=9), 19F (n=7), 15B (n=6) and 23B (n=6).

Among IPD isolates, the prevalence of serotypes included in the commercialized conjugate vaccines PCV7, PCV10 and PCV13 were 14.2% (65 isolates), 58.3% (267 isolates) and 83.6% (383 isolates) respectively. The prevalence of serotypes included in the three vaccines among isolates from the nasopharynx of healthy carriers was 23.6% (21 isolates), 27% (24 isolates) and 50.6% (45 isolates).

With respect to clonal properties the most frequent clonotypes among invasive isolates were ST306 (n=107), ST191 (n=31), ST1223 (n=25), ST304 (n=22), ST276 (n=17). A high variety of clonotypes were detected in carriers (56 different clonotypes in 89 strains); the most frequent being ST2372 (n=5), ST97 (n=4), ST42 (n=3), ST63 (n=3), ST180 (n=3), ST838 (n=3) and ST2690 (n=3). Finally, antibiotic susceptibility study was available in 543 of the 547 strains with 134 (24.5%) having diminished penicillin susceptibility (MIC ≥0.12).The percentage of isolates with diminished penicillin susceptibility was 23.4% (107 of 454) among invasive isolates and 30.3% (27 of 89) among carriers.

Overall prevalence of PcpA, *PsrP* and Pilus-1. The individual prevalence of *pcpA*, *psrP*, and Pilus-1 in the 547 strains of our collection were 92.7%, 51.2% and 15% without significant differences occurring between invasive and carrier isolates: for *pcpA* 92.8% vs. 92.1%; *P*=0.8, for *psrP* 51.7% vs. 48.3%; *P*=0.5 and for pilus-1 14.4% vs. 18%; *P* =0.3, respectively. Given the high prevalence of *pcpA* the potential coverage

with at least one protein of a multivalent vaccine including these three candidates would be high: 96.5% among invasive isolates (442 of 458 isolates) and 94.4% among carriers (84 of 89 isolates). Figure 1A shows the prevalence for each protein alone and for at least 1 of the proteins in the specific combinations (PcpA/PsrP/Pilus-1, PcpA/PsrP, PcpA/Pilus-1 and PsrP/Pilus-1). Notably, in Figure 1B, we show that 96% of the invasive isolates carried at least two of the three proteins, whereas 92% of the carrier isolates did the same. Likewise, 6% of isolates carried all 3 proteins, (4% and 8% of the invasive and carrier isolates, respectively). Thus, the majority of individuals immunized with a vaccine composed of these three antigens would have antibodies for at least 2 of these 3 proteins.

Prevalence based on clinical symptom and antibiotic resistance. The prevalence of *pcpA* among all strains was too high to have any correlation with any clinical condition. In contrast, the prevalence of *psrP* was significantly higher in patients with non complicated pneumonia (58.2; %*P*<.001) or empyema (59.5%; *P*<.001) than in children with bacteremia (40.4%). Inversely, the prevalence of pilus-1 was greater in patients with bacteremia than in patients with non-complicated (22.8% vs. 9.6%; P=0.005) and complicated pneumonia (11.7%; P=0.04) (Figure 2). We also observed significant differences in the prevalence of *psrP* and pilus-1 according to susceptibility for different antimicrobials (Table 1). Overall *psrP* was significantly more frequently detected in penicillin, cefotaxime, erythromycin and tetracycline susceptible isolates while pilus-1 and, to a modest level *pcpA*, were more frequently detected in isolates non susceptible to these antimicrobials. In contrast, *psrP* was significantly more frequently detected in chloramphenicol non-susceptible isolates.

Prevalence of *pcpA*, *psrP* and pilus-1 according to serotype and clonotype. Prevalence of these proteins was strongly associated with specific serotype and clonotypes. Table 2 shows significant differences in the prevalence of *pcpA*, *psrP* and pilus-1 according to serotype. *pcpA* is highly prevalent in almost all serotypes, the exception being serotype 3. *pcpA* was only detected in 7 of 21 isolates of serotype 3 (33.3%) vs. 500 of 526 non serotype 3 isolates (95.1%; P<.001). Interestingly, for

certain serotypes the prevalence of *psrP* was high but occurred with an absence of pilus-1 or vice versa. For example, the prevalence of *psrP* among 136 strains tested of serotype 1 was 80.1% (109 isolates) but pilus-1 was not detected in any strain of serotype 1. This observation was also detected for serotype 5 where *psrP* was detected in 88.2% of the 34 strains but Pilus-1 was absent. In contrast, for serotypes 14 or 6B the prevalence of *psrP* was significantly lower than the prevalence of pilus-1 (5.3% vs. 84.2% among serotype 14 isolates (n=19) and 35.7% vs. 57.1% among serotype 6B isolates (n=14). Other serotypes without pilus-1 included serotype 7F (none of 36 strains) and serotype 3 (none of 21 strains). *psrP* was also very low in these serotypes (11.1% for serotype 7F and 9.5% for serotype 3). In fact, of all 547 strains tested, only 4.2%, tested positive for both *psrP* and pilus-1.

Using the designation of serotypes having high or low attack rate [21, 22] *psrP* was significantly more frequent in serotypes categorized as having high attack rate (those less apt to be detected in carriage than in disease) than in serotypes categorized as low attack rate (those less apt to be detected in disease than in carriage) (58.7% vs. 39.1%; P<.001). *pcpA* was also more frequently detected in serotypes with high attack rate (95.6% vs. 87.5%; *P*=0.01). Pilus-1 distribution was similar in high and low attack rate serotypes (16.1% vs. 13.6%; *P*=0.4). Considering only penicillin susceptible isolates, the prevalence of *psrP* between high and low attack rate serotypes was different (72.3% vs. 44.9%; P<.001). The distribution of *pcpA* among these susceptible isolates was also higher in high attack rate serotypes vs. low attack rate serotypes (94.3% vs. 81.9%; P=0.01). Among penicillin susceptible isolates, the prevalence of Pilus-1 was higher in those expressing serotypes that are less apt to be detected in disease than in carriage (13.4% vs. 6.4%; P=0.02). Figure 3 shows the prevalence of PcpA, PsrP and Pilus-1 according to serotypes within the commercialized conjugate vaccines. Pilus-1 was more frequent detected among PCV7 serotypes vs. non PCV7 serotype 50% vs. 8.5%; P<001). In contrast, *psrP* was more frequent detected among non PCV7 isolates vs PCV7 isolates (53.8% vs. 37.2%; P=0.005).

Finally, we observed stark and significant differences in prevalence of these proteins according to clonotype among isolates expressing the same serotype (Table 3). *psrP* was detected in almost all ST306 (106 of 109 isolates; 97.2%) while practically in none

of the isolates with ST304 (1 of 22 isolates; 4.5%). Pilus-1 was totally absent in these clonotypes. The same phenomenon was observed for the penicillin susceptible clone ST1201: all isolates with this clone (n=19) have *psrP*, while none have Pilus-1. The opposite was observed for multiresistant clone ST320, which all (n=16) have pilus-1 yet lack *psrP*. Even in *pcpA*, which has a high prevalence within the entire collection, significant differences according to clonotype were detected in strains expressing the same serotype. For example, among isolates expressing serotype 3, *pcpA* was detected in 100% of strains with ST260, ST1220, ST1377 or ST2590 (6 isolates) while only in 6.6% of ST180 (1 of 15 isolates).

DISCUSSION

Among IPD isolates, the prevalence of disease caused by serotypes included in the commercialized conjugate vaccines increased from 14.2% in PCV7 to 83.6% in PCV13. In contrast, the overall prevalence of serotypes included in PCV13 in nasopharynx was only 50.6%. Thus, even though the newly introduced PCV13 vaccine had robust coverage against disease, its intermediate coverage of the current colonizing serotypes leaves open the possibility of serotype replacement by current invasive clones or continuing serotype shift. In the same way that an indirect effect of PCV7 preventing disease in adults and non-vaccinated children had been observed [5, 6], it is expected indirect protection offered by herd immunity using multivalent pneumococcal protein vaccines [26,27].

PcpA was highly prevalent in our collection, suggesting that it is a conserved pneumococcal component. While previous studies, including our own, have examined the prevalence of *psrP* or pilus-1 alone among clinical isolates [28-31], to our knowledge no information exists on the prevalence of *pcpA*. As indicated PcpA is an adhesin, and immunization with recombinant protein has been demonstrated to reduce the number of bacteria in the lungs of mice challenged with *S. pneumoniae* and to increase survival time in a mouse sepsis model following intraperitoneal challenge [19]. Most recently, PcpA has been shown to be required for *in vitro* biofilm formation [32], upregulated in response to Zn(2+) [33], and capable of eliciting antibodies during human nasopharyngeal colonization and acute otitis media [34], but not during bacteremia in infants [35]. Our finding that *pcpA* was present in 500 of the 526 serotypes, excluding serotype 3 isolates, underlines the importance of this protein for pneumococcal biology and strongly supports its inclusion in any protein vaccine.

Surprisingly, *pcpA* was only present in 7 of the 21 serotype 3 isolates tested. The absence of adhesins in serotype 3 isolates is not unprecedented; Choline binding protein A (CbpA; also known as PspC), which binds to both polymeric immunoglobulin receptor and laminin receptor, and has been implicated in biofilm formation, has a low prevalence within serotype 3 isolates [36]. Serotype 3 isolates are distinct from most other pneumococcal serotypes in that they are exceedingly encapsulated, and therefore

316 appear highly mucoid on blood agar plates. The absence of these adhesins and a
317 distinct clinical profile suggest that serotype 3 isolates might have a pathogenesis
318 dissimilar to other pneumococcal isolates, as numerous studies indicate that capsular
319 polysaccharide inhibits bacterial adhesion, and serotype 3 isolates are frequently
320 associated with necrotizing pneumonia. This suggests that a distinct protein vaccine
321 formulation would be required for protection against serotype 3-mediated disease. This
322 notion is supported by studies in experimentally infected mice, where a serotype 3
323 clinical isolate remained in the lungs but replicated to high titers, whereas clinical
324 isolates of serotype 2 and 4 replicated to lower titers but caused disseminated disease
325 [37].
326 PsrP is both an intraspecies and interspecies adhesin, mediating attachment to Keratin
327 10 on lung cells and promoting the presence of bacterial aggregates *in vivo* and biofilm
328 formation *in vitro* [38]. Pilus also functions as an adhesin, having been demonstrated to
329 mediate attachment to laminin and may also contribute to the invasiveness of strains
330 [39].
331 Importantly, considerable evidence indicates that immunization of mice with either the
332 basic region domain of PsrP or with individual components of Pilus-1 mediates
333 protection [16, 40]. Using Real-Time PCR, we detected *psrP* in 52.2% of all clinical
334 isolates, whereas we detected pilus-1 in 15% of all isolates. This was consistent with a
335 past study where the prevalence of *psrP* in clinical isolates was found to be 52.4% and
336 with studies of numerous other investigators where the prevalence of pilus-1 in clinical
337 isolates was found to be between 10-30% [30, 31, 41, 42].
338 Our study expands on these past studies by providing the prevalence of these
339 candidate vaccine antigens simultaneously. There by assessing the potential coverage
340 of a multivalent vaccine composed of *pcpA*, *psrP* and pilus-1. In all, 96% of the strains
341 examined carried at least 1 of these proteins, 96% carried 2, and 5% carried all 3. Our
342 analysis determined that *psrP* and pilus-1 have a negative correlation in multiple
343 serotypes raising the possibility that *psrP* and pilus-1 may have redundant roles, or that
344 their production might be metabolically expensive and that an individual strain cannot
345 support production of both of these extremely large proteins. Briefly, PsrP is a
346 glycosylated surface protein that separates at a molecular weight >2000 kDa, whereas

Pilus-1 is primarily composed of multiple repeats of the subunit RrgB. Both extend beyond the bacterial capsule to mediate adhesion. Interestingly, our study shows that PsrP was found significantly among serotypes that are less apt to be detected in carriage than in disease, while Pilus-1 was not associated with these virulent serotypes. These data could suggest that PsrP is in part responsible for the increased virulence of high attack rate serotypes. Along this line, it is known that variation in virulence exists among isolates of the same serotype, due to the contribution of serotype-independent factors associated with clonal type [43]. The variability of the prevalence of *pcpA*, *psrP* and pilus-1 according to clonal type in strains expressing the same serotype confirms that the presence of these factors appears to be a clonal property. This fact has been reported for Pilus-1 by other authors [41].

Antibiotic resistance was associated with the presence of pilus-1 and showed a negative correlation with *psrP*. The association of pilus-1 with antibiotic resistance has been reported previously, but the reasons for this association are not clear. It could be that the *rrlA* islet and specific resistance genes might be recombined together. Moschioni et al. suggest that pilus aid in adhesion during colonization of the nasopharynx and that pilus expressing strains could be selected as a result of antibiotic treatment [44]. The reason for negative association of PsrP with resistant strains is unknown. Interestingly, PsrP had greater correlation with strains isolated from individuals with pneumonia, both uncomplicated and complicated, whereas Pilus-1 had a predilection for strains associated with bacteremia. This observation is consistent with the known roles of PsrP as a lung cell adhesin and Pilus-1 as a mediator of invasive disease [17].

A limitation of the study is that the absence or presence of these genes/proteins is based on PCR results of wellknown and published genes [15, 18, 44] but potential primer divergence could implied that a PCR negative result is not necessary equivalent of the absence of the protein and viceversa.

In summary, our results indicate that *pcpA* is highly prevalent and its addition to a multivalent pneumococcal protein vaccine would result in considerable coverage. In

contrast, *psrP* and pilus-1 have less robust individual coverage but, since *psrP* is present in high attack rate strains and pilus-1 in antibiotic resistant strains, could be added in an effort to reduce the likelihood of disease. The inverse correlation of these proteins suggests that they could be paired as part of a multi-valent vaccine to compensate for each other. This notion is highlighted by the fact that 96% of all strains carried *pcpA* and either *psrP* or pilus 1. Future studies are planned to determine the protective efficacy of this trivalent vaccine against invasive disease caused by multiple clinical isolates.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: CJO, CMA. Performed the experiments: LS, EdA. Analyzed the data: LS, RP, CMA. Contributed reagents/materials/analysis tools: KB, CJO,PC. Wrote the paper: LS, RP, CJO, CMA. Revised the paper: all authors

413 REFERENCES

414 1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, et al. (2009) Burden of
415 disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global
416 estimates. Lancet 374:893-902.
417
418 2. Obando I, Muñoz-Almagro C, Arroyo LA, Tarrago D, Sanchez-Tatay D, et al. (2008)
419 Pediatric parapneumonic empyema, Spain. Emerg Infect Dis 14:1390-1397.
420
421 3. Li ST, Tancredi DJ (2010) Empyema hospitalizations increased in US children
422 despite pneumococcal conjugate vaccine. Pediatrics125:26-33.
423
424 4. Byington CL, Korgenski K, Daly J, Ampofo K, Pavia A, Mason EO (2006) Impact of
425 the pneumococcal conjugate vaccine on pneumococcal parapneumonic empyema.
426 Pediatr Infect Dis J 25:250-254.
427
428 5. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, et al; Active Bacterial Core
429 Surveillance/Emerging Infections Program Network (2010) Sustained reductions in
430 invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 201:32-41.
431
432 6. Pulido M, Sorvillo F (2010) Declining invasive pneumococcal disease mortality in the
433 United States, 1990-2005. Vaccine 28:889-892.
434
435 7. Eastham KM, Freeman R, Kearns AM, Eltringham G, Clark J et al. (2004) Clinical
436 features, aetiology and outcome of empyema in children in the north east of England.
437 Thorax 59:522-525.
438
439 8. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, et al. (2008)
440 Emergence of invasive pneumococcal disease caused by non-vaccine serotypes in the
441 era of 7-valent conjugate vaccine. Clin Infect Dis 46:174-182.
442

9. Ardanuy C, Tubau F, Pallares R, Calatayud L, Domínguez MA, et al. (2009) Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997-2007. Clin Infect Dis 48:57-64.

10. Tarragó D, Aguilar L, García R, Gimenez MJ, Granizo JJ, et al. (2011) Evolution of clonal and susceptibility profiles of serotype 19A *Streptococcus pneumoniae* among invasive isolates from children in Spain, 1990 to 2008. Antimicrob Agents Chemother 55:2297-2302.

11. Grijalva CG, Poehling KA, Nuorti JP, Zhu Y, Martin SW, et al. (2006) National impact of universal childhood immunization with pneumococcal conjugate vaccine on outpatient medical care visits in the United States. Pediatrics 118:865-873.

12. Huang SS, Johnson KM, Ray GT, Wroe P, Lieu TA, et al. (2011) Healthcare utilization and cost of pneumococcal disease in the United States. Vaccine 29:3398-3412.

13. Pneumonia. Strategy overview. Bill and Melinda Gates Foundation. Available: http://www.gatesfoundation.org/global-health/Documents/pneumonia-strategy.pdf Accessed 2011 Dec 1

14. Pneumococcal vaccine support. Gavi Alliance. Available: http://www.gavialliance.org/support/nvs/pneumococcal/ Accessed 2011 Dec 1

15. Obert C, Sublett J, Kaushal D, Hinojosa E,Barton T, et al. (2006) Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. Infect Immun 74:4766-4777.

16. Rose L, Shivshankar P, Hinojosa E, Rodriguez A, Sanchez CJ, et al. (2008) Antibodies against PsrP, a novel *Streptococcus pneumoniae* adhesin, block adhesion and protect mice against pneumococcal challenge. J Infect Dis198:375-383.

17. Barocchi MA, Ries J, Zogaj X, Hemsley C, Albiger B, et al. (2006) A pneumococcal pilus influences virulence and host inflammatory responses. Proc Natl Acad Sci U S A 103:2857-2862.

18. Sánchez-Beato AR, López R, García JL (1998) Molecular characterization of PcpA: a novel choline-binding protein of *Streptococcus pneumoniae*. FEMS Microbiol Lett 164:207-214.

19. Glover DT, Hollingshead SK, Briles DE (2008) *Streptococcus pneumoniae* Surface Protein PcpA Elicits Protection against Lung Infection and Fatal Sepsis. Infect and Immunity 76:2767–2776.

20. Tarrago D, Fenoll A, Sanchez-Tatay D, Arroyo LA, Muñoz-Almagro C, et al. (2008) Identification of pneumococcal serotypes from culture-negative clinical specimens by novel Real-Time PCR. Clin Microbiol Infect 14:828-834.

21. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, et al. (2003) Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 187:1424-1432.

22. Sleeman KL, Griffiths D, Shackley F (2006) Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. J Infect Dis 194:682-688.

23. National Committee for Clinical Laboratory Standards (NCCLS) (2008). Performance Standards for antimicrobial susceptibility testing: Eighteenth informational

503 supplement. CLSI document M100-S18 (ISBN 1-5-56238-653-0) Clinical and laboratory
504 standard institute. Wayne Pa
505
506 24. Enright MC, Spratt BG (1998) A multilocus sequence typing scheme for
507 *Streptococcus pneumoniae*: identification of clones associated with serious invasive
508 disease. Microbiology 144:3049-3060.
509
510 25. Muñoz-Almagro C, Gala S, Selva L, Jordan I, Tarragó D, et al. (2011) DNA bacterial
511 load in children and adolescents with pneumococcal pneumonia and empyema. Eur J
512 ClinMicrobiol Infect D 30:327-335.
513
514 26. Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, et al. (2000). Intranasal
515 immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is
516 highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect
517 Immun 68:796-800.
518
519 27. Tai SS (2006) *Streptococcus pneumoniae* Protein Vaccine Candidates: Properties,
520 Activities and Animal Studies. Crit Rev Microbiol 32:139-53.
521
522 28. Muñoz-Almagro C, Selva L, Sanchez CJ, Esteva C, de Sevilla MF, et al. (2010)
523 PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia
524 and is strongly associated with clonal type. Clin Vaccine Immunol 17:1672-1678.
525
526 29. Imai S, Ito Y, Ishida T, Hirai T, Ito I, et al; Kansai Community Acquired
527 Pneumococcal Pneumonia Study Group (2011) Distribution and clonal relationship of
528 cell surface virulence genes among *Streptococcus pneumoniae* isolates in Japan. Clin
529 Microbiol Infect 17:1409-1414.
530
531 30. Vainio A, Kaijalainen T, Hakanen AJ, Virolainen A (2011) Prevalence of pilus-
532 encoding islets and clonality of pneumococcal isolates from children with acute otitis
533 media. Eur J Clin Microbiol Infect Dis 30:515-519.

534
535 31. Moschioni M, De Angelis G, Melchiorre S, Masignani V, Leibovitz E, et al. (2010)
536 Prevalence of pilus-encoding islets among acute otitis media *Streptococcus*
537 *pneumoniae* isolates from Israel. Clin Microbiol Infect 16:1501-1504.
538
539 32. Moscoso M, García E, López R (2006) Biofilm formation by *Streptococcus*
540 *pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in
541 microbial accretion. J Bacteriol188:7785-7795.
542
543 33. Kloosterman TG, Witwicki RM, van der Kooi-Pol MM, Bijlsma JJ, Kuipers OP (2008)
544 Opposite effects of Mn2+ and Zn2+ on PsaR-mediated expression of the virulence
545 genes pcpA, prtA, and psaBCA of *Streptococcus pneumoniae*. J Bacteriol 190: 5382-
546 5393.
547
548 34. Kaur R, Casey JR, Pichichero ME (2011) Serum antibody response to five
549 *Streptococcus pneumoniae* proteins during acute otitis media in otitis-prone and non-
550 otitis-prone children. Pediatr Infect Dis J 30:645-650.
551
552 35. Hagerman A, Posfay-Barbe KM, Grillet S, Ochs MM, Brookes RH, et al. (2011)
553 Failure to elicit seroresponses to pneumococcal surface proteins (pneumococcal
554 histidine triad D, pneumococcal choline-binding protein A, and serine proteinase
555 precursor A) in children with pneumococcal bacteraemia. Clin Microbiol Infect. In press.
556 doi: 10.1111/j.1469-0691.2011.03629.x.
557
558 36. Brooks-Walter A, Briles DE, Hollingshead SK (1999) The pspC gene of
559 *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-
560 reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. Infect
561 Immun 67: 6533-6542.
562
563 37. Orihuela CJ, Gao G, McGee M, Yu J, Francis KP, et al. (2003) Organ-specific
564 models of *Streptococcus pneumoniae* disease. Scand J Infect Dis 35:647-652.

565
566 38. Sanchez CJ, Shivshankar P, Stol K, Trakhtenbroit S, Sullam PM, et al. (2010) The
567 pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that
568 promotes bacterial aggregation in vivo and in biofilms. PLoS Pathog 6: e1001044.
569
570 39. Hilleringmann M, Giusti F, Baudner BC, Masignani V, Covacci A, et al. (2008)
571 Pneumococcal pili are composed of protofilaments exposing adhesive clusters of Rrg A.
572 PLoS Pathog 4:e1000026
573
574 40. Harfouche C, Filippini S, Gianfaldoni C, Ruggiero P, Moschioni M, et al. (2012).
575 RrgB321, a fusion protein of the three variants of the pneumococcal pilus backbone
576 RrgB, is protective in vivo and elicits opsonic antibodies. Infect Immun 80:451-460.
577
578 41. Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M (2008) The presence of
579 the pilus locus is a clonal property among pneumococcal invasive isolates. BMC
580 Microbiol 8: 41.
581
582 42. Regev-Yochay G, Hanage WP, Trzcinski K, Rifas-Shiman SL, Lee G, et al. (2010)
583 Re-emergence of the type 1 pilus among *Streptococcus pneumoniae* isolates in
584 Massachusetts, USA. Vaccine 28: 4842-4846.
585
586 43. Harvey RM, Stroeher UH, Ogunniyi AD, Smith-Vaughan HC, Leach AJ, et al. (2011)
587 A variable region within the genome of *Streptococcus pneumoniae* contributes to strain-
588 strain variation in virulence. PLoS One 6: e19650.
589
590 44. Moschioni M, Donati C, Muzzi A, Masignani V, Censini S, et al. (2008)
591 *Streptococcus pneumoniae* contains 3 rlrA pilus variants that are clonally related. J
592 Infect Dis197: 888-896.
593

FIGURE LEGENDS

Figure 1. Prevalence of PcpA, PsrP and Pilus**.** (A) Prevalence for PcpA, PsrP and Pilus alone and for their combinations (isolates with al least one of the three combinations) in 458 pneumococcal isolates of patients with invasive pneumococcal disease (IPD) and in 89 pneumococcal isolates of healthy nasopharyngeal carriers. (B) Prevalence of strains that carry all three proteins, and two of possible protein combinations including PcpA and PsrP, Psrp and Pilus-1, PcpA and Pilus-1 among pneumococcal isolates of patients with IPD and healthy nasopharyngeal carriers.

Figure 2. Prevalence of PcpA, PsrP and Pilus according to clinical syndrome among pneumococcal invasive isolates.

Figure 3. Prevalence of PcPA, PsrP and Pilus according to serotypes within the commercialized conjugate vaccines.

611 TABLES

612

613 Table 1. Prevalence of PcpA, PsrP and Pilus according to antimicrobial susceptibility.

614

Antimicrobial agent			PcpA		PsrP		Pilus	
MIC	Number of isolates	%	% of positive	P	% of positive	P	% of positive	P
Penicillin								
≤ 0.06	409	75.3	90.2	<.000	62.6	<.000	8.8	<.001
≥ 0.12	134	24.7	100		17.9		34.3	
Cefotaxime								
≤ 0.5	482	88.8	91.7	0.01	56.4	<.000	9.3	<.001
≥ 1	61	11.2	100		13.1		60.7	
Erythromicine								
≤ 0.25	415	76.4	90.6	0.001	58.3	<.000	10.8	<.001
≥ 0.5	128	23.6	99.2		29.7		28.9	
Tetracycline*								
≤ 2	409	75.9	91.4	0.07	58.9	<.000	11.2	0.001
≥ 4	130	24.1	96.2		28.5		27.7	
Chloramphenicol**								
≤ 4	515	95.2	92.8	0.4	50.7	0.02	15.7	0.09
≥ 8	26	4.8	88.5		73.1		3.8	

615

616 The study was non-available in four* and six** isolates

617

618 Table 2. Prevalence of PcpA,PsrP and Pilus according to serotype of isolates.

619

Serotype	Number of Isolates	PcpA Positive	%	PsrP Positive	%	Pilus Positive	%
Overall	547	507	92.7	280	51.2	82	15.0
1	136	132	97.1	109	80.1	0	0.0
19A	93	90	96.8	44	47.3	26	28.0
7F	36	36	100.0	4	11.1	0	0.0
5	34	28	82.4	30	88.2	0	0.0
6A	22	21	95.5	11	50.0	4	18.2
3	21	7	33.3	2	9.5	0	0.0
19F	20	16	80.0	13	65.0	6	30.0
14	19	19	100.0	1	5.3	16	84.2
6B	14	14	100.0	5	35.7	8	57.1
15B	13	13	100.0	10	76.9	2	15.4
9V	12	12	100.0	3	25.0	9	75.0
23B	12	12	100.0	1	8.3	0	0.0
24F	10	10	100.0	1	10.0	0	0.0
23F	10	10	100.0	1	10.0	0	0.0
10A	9	9	100.0	2	22.2	1	11.1
23A	6	6	100.0	4	66.7	0	0.0
18C	6	6	100.0	5	83.3	0	0.0
15C	6	6	100.0	5	83.3	1	16.7
38	6	3	50.0	2	33.3	2	33.3
21	5	5	100.0	3	60.0	0	0.0
4	5	3	60.0	4	80.0	4	80.0
15A	4	4	100.0	1	25.0	0	0.0
24	4	4	100.0	2	50.0	0	0.0
35B	3	3	100.0	1	33.3	1	33.3
22F	3	3	100.0	3	100.0	0	0.0
16F	3	3	100.0	3	100.0	0	0.0

12F	3	3	100.0	0	0.0	0	0.0
9N	2	2	100.0	1	50.0	0	0.0
37	2	2	100.0	0	0.0	0	0.0
34	2	1	50.0	0	0.0	1	50.0
31	2	2	100.0	0	0.0	0	0.0
29	2	2	100.0	1	50.0	0	0.0
28	2	1	50.0	0	0.0	0	0.0
27	2	1	50.0	0	0.0	0	0.0
22	2	2	100.0	1	50.0	0	0.0
16	2	2	100.0	2	100.0	0	0.0
6C	1	1	100.0	0	0.0	0	0.0
35F	1	1	100.0	1	100.0	0	0.0
33F	1	1	100.0	0	0.0	0	0.0
24B	1	1	100.0	0	0.0	0	0.0
17F	1	1	100.0	1	100.0	0	0.0
11A	1	1	100.0	0	0.0	0	0.0
47	1	1	100.0	0	0.0	1	100.0
39	1	1	100.0	1	100.0	0	0.0
17	1	1	100.0	0	0.0	0	0.0
13	1	1	100.0	1	100.0	0	0.0
11	1	1	100.0	1	100.0	0	0.0
10	1	1	100.0	0	0.0	0	0.0
8	1	1	100.0	0	0.0	0	0.0
2	1	1	100.0	0	0.0	0	0.0

623 Table 3. Prevalence of PcpA. PsrP and Pilus according to clonotypes (ST) detected in
624 the study.
625

ST	Number of Isolates	Serotype	PcpA positive	%PcpA positive	PsrP positive	% PsrP positive	Pilus positive	% Pilus Positive
306	109	1 (n=109)	107	98.2	106	97.2	0	0.0
191	32	7F (n=32)	32	100.0	3	9.4	0	0.0
1223	25	5 (n=25)	23	92.0	21	84.0	0	0.0
304	22	1 (n=22)	22	100.0	1	4.5	0	0.0
1201	19	19A (n=19)	18	94.7	19	100.0	0	0.0
276	18	19A (n=18)	18	100.0	3	16.7	0	0.0
320	16	19A (n=16)	16	100.0	0	0.0	16	100.0
180	15	3 (n=15)	1	6.7	1	6.7	0	0.0
156	13	14 (n=13)	13	100.0	0	0.0	13	100.0
2013	13	19A (n=13)	13	100.0	2	15.4	0	0.0
2372	12	23B (n=10)	10	83.4	1	8.3	0	0.0
		19A (n=1)	1	8.3	1	8.3	1	8.3
		23F (n=1)	1	8.3	0	0	0	0.0
97	11	10A (n=11)	11	100.0	2	18.2	1	9.1
289	8	5 (n=8)	4	50.0	8	100.0	0	0.0
63	7	15A (n=4)	4	57.1	1	14.3	0	0.0
		15B (n=1)	1	14.3	1	14.3	0	0.0
		15C (n=1)	1	14.3	0	0.0	0	0.0
		38 (n=1)	1	14.3	1	14.3	0	0.0
4677	6	24F (n=6)	6	100.0	0	0.0	0	0.0
2100	6	19F (n=6)	6	100.0	1	16.7	0	0.0
1167	6	19F (n=5)	1	16.7	5	83.3	4	66.6
		19A (n=1)	0	0.0	1	16.7	1	16.7
838	6	9V (n=6)	6	100.0	0	0.0	6	100.0
230	6	24F (n=3)	3	50.0	0	0.0	0	0.0
		24 (n=2)	2	33.3	0	0.0	0	0.0

		24B (n=1)	1	16.7	0	0.0	0	0.0
202	6	19A (n=6)	5	83.3	2	33.3	5	83.3
113	6	18C (n=6)	6	100.0	5	83.3	0	0.0
199	5	19A (n=4)	4	80.0	4	80.0	0	0.0
		15B (n=1)	1	20.0	1	20.0	0	0.0
42	5	23A (n=5)	5	100.0	4	80.0	0	0.0
1262	4	15B (n=2)	2	50.0	2	50.0	0	0.0
		15C (n=2)	2	50.0	2	50.0	0	0.0
433	4	22 (n=1)	1	25.0	0	0.0	0	0.0
		22F (n=1)	1	25.0	1	25.0	0	0.0
		19A (n=1)	1	25.0	0	0.0	0	0.0
		28 (n=1)	1	25.0	0	0.0	0	0.0
416	4	19A (n=4)	4	100.0	4	100.0	1	25.0
386	4	6B (n=4)	4	100.0	1	25.0	2	50.0
90	4	6A (n=2)	1	25.0	0	0.0	2	50.0
		6B (n=2)	2	50.0	0	0.0	2	50.0
81	4	19A (n=2)	2	50.0	2	50.0	0	0.0
		19F (n=1)	1	25.0	1	25.0	0	0.0
		23F (n=1)	1	25.0	1	25.0	0	0.0
30	4	16 (n=2)	2	50.0	2	50.0	0	0.0
		16F (n=2)	2	50.0	2	50.0	0	0.0
2690	3	29 (n=2)	2	66.7	1	33.3	0	0.0
		21 (n=1)	1	33.3	0	0.0	0	0.0
1684	3	31 (n=2)	2	66.7	0	0.0	0	0.0
		1 (n=1)	1	33.3	0	0.0	0	0.0
1143	3	6A (n=3)	3	100.0	3	100.0	1	33.3
310	3	38 (n=2)	0	0.0	0	0.0	2	66.7
		34 (n=1)	0	0.0	0	0.0	1	33.3
280	3	9V (n=2)	2	66.7	2	66.7	0	0.0
		9N (n=1)	1	33.3	1	33.3	0	0.0
224	3	6A (n=3)	3	100.0	0	0.0	0	0.0

193	3	21 (n=2)	2	66.7	2	66.7	0	0.0
		15B (n=1)	1	33.3	1	33.3	0	0.0
101	3	15C (n=2)	2	66.7	2	66.7	0	0.0
		15B (n=1)	1	33.3	0	0.0	0	0.0
72	3	24 (n=2)	2	66.7	2	66.7	0	0.0
		24F (n=1)	1	33.3	1	33.3	0	0.0

Other ST detected with 2 isolates each: ST62, ST109, ST162, ST177, ST338, ST393, ST439, ST447, ST558, ST989, ST1011, ST1220, ST1377, ST1624, ST1692, ST2611, ST2948, ST4310, ST4828, ST5223, and ST5740.

1 isolate each: ST9, ST66, ST88, ST94, ST110, ST124, ST143, ST176, ST179, ST205, ST217, ST228, ST245, ST260, ST274, ST311, ST315, ST327, ST343, ST392, ST404, ST425, ST446, ST450, ST460, ST494, ST557, ST614, ST876, ST994, ST1012, ST1064, ST1264, ST1475, ST1504, ST1577, ST1589, ST1611, ST1664, ST1844, ST1848, ST2319, ST2333, ST2376, ST2377, ST2467, ST2557, ST2590, ST2592, ST2594, ST2595, ST2618, ST2946, ST2947, ST2949, ST3254, ST3259, ST3436, ST3437, ST3438, ST3490, ST3609, ST3787, ST4306, ST4676, ST4796, ST4826, ST4832, ST4834, ST5224, ST5741, ST5825, ST5829, ST6006, ST6040, ST6394, ST6518 and ST6519.

Clinical Presentation of Invasive Pneumococcal Disease in Spain in the Era of Heptavalent Conjugate Vaccine

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Background: The aim of this study was to analyze the rate of incidence, clinical presentation, serotype, and clonal distribution of invasive pneumococcal disease (IPD) in the era of heptavalent pneumococcal conjugate vaccine (PCV7) in Barcelona, Spain.

Methods: This was a prospective study comprising all children <5 years with IPD who were managed in 2 tertiary-care, pediatric hospitals between January 2007 and December 2009. IPD was defined as the presence of clinical findings of infection together with isolation or detection of DNA of *Streptococcus pneumoniae* in a sterile fluid sample.

Results: In this study, 319 patients (53.3% male), mean age 29.6 months, were included. Comparing rates in 2007 and 2009 (76.2 and 109.9 episodes/100,000 population, respectively), an increase of 44% (95% confidence interval, 10%–89%) was observed. The main clinical presentation was pneumonia (254 episodes, 79.6%), followed by meningitis (29, 9.1%), and bacteremia (25, 7.8%).The diagnosis was made by positive culture in 123 (38.6%) patients and in 196 (61.4%) by real-time polymerase chain reaction. Serotype study was performed in 300 episodes, and 273 (91%) were non-PCV7 serotypes. The most frequent serotypes were 1 (20.7%), 19A (15.7%), and 3 (12.3%). A minimal inhibitory concentration ≥0.12 μg/mL to penicillin was detected in 34.4% of isolates. Sequence type 306 expressing serotype 1 was the most frequent clonal type detected (20.3% of studied strains).

Conclusions: IPD continues to increase in Barcelona, and the rate is higher than previously reported as a result of low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes and pneumonia was the main clinical presentation.

Key Words: *Streptococcus pneumoniae*, pneumococcal conjugate vaccine, pneumonia, serotype, MLST

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Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among young children, despite the availability of antibiotic treatment and vaccines. The World Health Organization estimates that every year more than one million children younger than 5 years die of invasive pneumococcal disease (IPD), mainly in developing countries.[1]

The imbalance between host factors and virulence of the pathogen is partly responsible for the production of IPD. The main virulence factor of pneumococcus is the polysaccharide capsule, with 93 serotypes with differing pathogenicity.[2]

Following introduction of pneumococcal conjugate vaccine (PCV7) in the United States, there was a dramatic decline in IPD rates and drug-resistant pneumococci.[3,4] However, in Spain and other countries, we observed a significant increase in the rate of IPD caused by non-PCV7 serotypes and a slight reduction in the rate of IPD caused by PCV7 serotypes.[5] There was a change in the main serotypes associated with IPD, and this change was associated with changes in clinical types of IPD,[6] a reduction in the rate of antibiotic-resistant strains causing IPD, and the emergence of previously established virulent clones of non-PCV7 serotypes.[5]

The introduction of real-time polymerase chain reaction (PCR)-based methods that specifically identify capsular DNA in direct sample offer a sensitive, rapid, and simple approach for the surveillance of IPD.[7] Different authors have reported that molecular methods can be used directly on sterile biologic samples, improving the ability to diagnose IPD.[8–12] At present, little is known about the epidemiologic characteristics, clinical presentation, and outcome of IPD including episodes with negative bacterial culture. The purpose of this study was to determine the epidemiologic variables, clinical presentation, current trends, and serotypes and clones of *S. pneumoniae* among children in Barcelona, Spain, after the implementation of PCV7, in 2001, including patients with negative culture who were diagnosed by real-time PCR.

MATERIALS AND METHODS

Patients and Definitions

We performed a prospective study comprising all children <5 years with IPD managed in 2 tertiary-care pediatric hospitals in Barcelona (Spain) during a 3-year period (January 2007-December 2009). These 2 centers serve a pediatric referral population of 134,662 children <5 years (around 27% of the Catalan pediatric population <5 years).[13]

An episode of IPD was defined as the presence of clinical findings of infection together with isolation and/or DNA detection of pneumolysin *(ply)* gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any sterile body fluid such as blood, cerebrospinal fluid, pleural fluid, or articular fluid.

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Data Collected and Analyzed

Epidemiologic characteristics included age, gender, immunization status against *S. pneumoniae* (when written records were available), underlying medical condition, group child care attendance, antibiotic treatment and/or respiratory infection before the diagnosis of IPD, history of breast-feeding, household size, and exposure to tobacco smoke.

Clinical characteristics including clinical presentation, intensive care unit (ICU) admission, complications, antibiotic treatment and duration, days of hospitalization, and clinical outcome were also recorded.

Microbiologic Bacterial Culture and Antimicrobial Susceptibility Studies

All pneumococcal isolates were identified by standard microbiologic methods that remained constant during the study period. Agar dilution technique was used to determine the minimal inhibitory concentration (MIC) of several antibiotics, including penicillin and cefotaxime. American Type Culture Collection 49619 (serotype 19F) was used as a control. Susceptibility to penicillin and other antibiotics was defined according to the 2008 meningeal break points by the Clinical Laboratory Standards Institute.[14] Isolates with intermediate- or high-level resistance were defined as nonsusceptible.

DNA Detection of *S. pneumoniae* by Real-time PCR

Detection of *ply* gene of *S. pneumoniae* was performed by real-time PCR according to a published assay.[7]

Serotype Identification

Serotyping of strains isolated by culture was carried out by the Quellung reaction, using antisera provided by the Statens Serum Institut (Copenhagen, Denmark), or by dot-blot serotyping.[15] MICs and serotyping of the strains were performed at the National Center for Microbiology (Majadahonda, Spain). Detection of pneumococcal serotypes in negative culture clinical samples but *ply* pneumococcal gene positive was performed according to a published multiplex real-time PCR methodology.[16] This procedure includes the DNA detection of conserved *wzg* capsular gene of *S. pneumoniae* and other different genes selected to distinguish 24 serotypes (1, 3, 4, 5, 6A/C, 6B/D, 7F/A, 8, 9V/A/N/L, 14, 15B /C, 18C/B, 19A, 19F/B/C, 23A, and 23F).

Serotypes were classified into the following groups: PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F), PCV10 serotypes (PCV7 serotypes plus 1, 5, 7F), and PCV13 serotypes (PCV10 serotypes plus 19A, 6A, 3).

Clonal Study

Clonal composition of strains was analyzed using multilocus sequence typing (MLST) as reported elsewhere.[17] The assignment of alleles and sequence types (ST) was carried out using the software at the pneumococcal web page www.mlst.net. Analysis of ST and assignment to clonal complex (CC) were performed with the eBURST program.[18] STs that shared 6 of 7 alleles (single locus variants) were considered a CC.

Statistical Analysis

Rates of IPD, defined as the number of episodes per 100,000 population, were calculated using the annual estimates of pediatric population obtained from the Department of Statistics in Catalonia[13] and the percentage of capture of both hospitals among total hospitalization in children <5 years. In Catalonia county, these hospitals captured, during the study period, 25.4% of all pediatric hospitalizations <2 years and 32.2% of pediatric hospitalizations between 2 to 5 years.

We used the χ^2 test or Fisher exact test to compare proportions and Student *t* test to compare means. Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS), and Epi Info, version 6.0 (Centers for Disease Control and Prevention). We calculated 95% confidence intervals (CIs), and 2-sided *P* values ≤0.05 were considered to be statistically significant.

RESULTS

During the study period, 319 episodes of IPD were identified in 319 patients, including 170 male patients (53.3%) and 149 female patients (46.7%), with a mean age of 29.6 months (standard deviation [SD]: 15.7). One hundred ninety-two episodes (60.2%) were in children aged 24 to 59 months, 99 (31.0%) in children aged 7 to 23 months, and 28 (8.8%) in children <6 months of age.

There was clearly seasonal variation. 73% of episodes were detected during cool months (October to March) versus 27.2% during warm months (April-September), $P < 0.001$.

Two hundred forty-four (76.5%) patients reported group child care attendance, 144 (45.1%) patients had a viral respiratory infection by history during the month before IPD, and 44 (13.8%) had received antibiotic treatment the month before IPD.

Two hundred twenty-five (70.5%) patients reported a history of breast-feeding, and 122 (38.2%) patients had been exposed to tobacco smoke. The mean household size was 4 cohabitants (SD: 1.2, range: 2–10).

According to the criteria of the American Academy of Pediatrics,[19] only 5 of 319 (1.5%) children were at high risk of IPD, including 2 children with malignant disease who were receiving immunosuppressive therapy, 1 with diabetes mellitus, 1 with congenital cyanotic cardiopathy, and 1 with pulmonary emphysema.

Concerning immunization status for *S. pneumoniae*, 168 (52.8%) cases had received at least 1 dose of PCV7, although only 141 (44.3%) were considered fully vaccinated by age.

Incidence

Rates of IPD increased between 2007 and 2009; the incidence of IPD in <5 years in 2007 was 76.2 cases/100,000 population; in 2008, it was 82.2 cases/100,000 population; and in 2009, it was 109.9 cases/100,000 population. Comparing rates in 2007 and 2009, there was an increase of 44% (95% CI: 10%–89%; $P = 0.008$). There was a significant increase in the rate of pneumonia during the study period: an increase of 81% (95% CI: 33%–148%; $P = 0.001$) comparing 2007 versus 2009. There were no significant changes in the rates of meningitis and bacteremia during the study (Table, Supplemental Digital Content 1, http://links.lww.com/INF/B14 shows rate of IPD in children according to age group during 2007–2009).

Clinical Presentation

Overall, the clinical diagnosis of patients included in this study was pneumonia in 254 (79.6%) patients, meningitis in 29 (9.1%), bacteremia in 25 (7.8%), arthritis or osteomyelitis in 6 (1.9%), sepsis in 3 (0.9%), and cellulitis in 2 (0.6%). Among pneumonia cases, 51 (20.1%) were noncomplicated pneumonia, 171 (67.3%) were empyema, and 32 (12.6%) parapneumonic pleural effusion.

Table 1 shows the distribution of positive samples detected by culture and by real-time PCR according to main clinical presentations.

Children were admitted to the hospital for 310 (97.2%) of the 319 episodes. The mean length of stay was 10.8 days (SD: 7.5). The longest mean stay by clinical presentation was 18.25 days (SD: 13.19) for meningitis. Of note, patients with noncomplicated pneumonia have no statically differences in the median age,

TABLE 1. Distribution of Positive Samples Detected by Culture and by Real-time PCR According to Main Clinical Presentations

	Positive Blood Culture	Positive Plasma Real–time PCR	Positive Pleural Effusion Culture	Positive Pleural Effusion Real–time PCR
Noncomplicated Pneumonia (n = 51)	18	39		
Parapneumonic pleural effusion (n = 32)	5	18	0	12
Empyema (n = 171)	18	75	32	151
			CSF Culture	CSF Real–Time PCR
Meningitis (n = 29)	15	18	20	19
Bacteremia (n = 25)	25	2		

gender, days of hospitalization, and total days of antibiotic in the groups "positive blood culture" and "only plasma real-time PCR positive."

The mean of days of antibiotic therapy (including extrahospital treatment) was 17.8 days (SD: 6.8). Arthritis and osteomyelitis were the diagnosis with the longest duration of antibiotic therapy (28.17 days, SD: 11.78).

Forty-four children (13.8%) were admitted to the pediatric intensive care unit. Overall, 27 of 29 episodes of meningitis (93.1%), 14 of 254 (5.5%) episodes of pneumonia, and 3 of 3 (100%) episodes of sepsis were admitted to ICU.

Among children admitted to ICU, 22 (51.2%) had received at least 1 dose of PCV7, but only 19 (44.2%) were fully vaccinated for age.

Of the 319 patients, there were 4 (1.3%) deaths, 3 patients with meningitis and 1 with sepsis. Thirty-four patients (10.7%) had sequelae associated with *Streptococcus pneumoniae*: neurologic sequelae in 15 of 29 (51.7%) meningitis episodes and pulmonary sequelae in 17 of 254 (6.7%) children with pneumonia.

Serotypes, Molecular Study, and Antibiotic Susceptibility

Diagnosis was established in 123 (38.6%) episodes by culture and in 196 (61.4%) by real-time PCR.

The serotyping study was done in 300 (94%) of the total IPD episodes. In 120 (40%), the serotyping study was carried out with strain isolates from culture, whereas 180 (60%), were done with direct samples by multiplex, real-time PCR. Overall, 23 different serotypes were identified. Nevertheless, there was a large number (76, 25.3%) of samples with *ply* and *wzg* gene positive but no specific gene of 24 serotypes tested, so we considered these as "other serotypes." The most frequent among identified serotypes were serotype 1 (62; 20.7%), 19A (47; 15.7%), and 3 (37; 12.3%). Of the 300 episodes, 27 (9%) were caused by PCV7 serotypes and 273 (91%) were caused by non-PCV7 serotypes. One-hundred nineteen (39.7%) were caused by PCV10 serotypes and 209 (69.7%) by PCV13 serotypes. Of 27 patients who had IPD attributed to PCV7 serotypes, 5 were well vaccinated. The characteristics of vaccinated children with IPD caused by PCV7 serotypes are shown in Table 2.

There were significant differences in the clinical presentation among the most prevalent serotypes detected in the study: serotype 1 and serotype 3 were significantly associated with pneumonia, whereas the clinical presentation of episodes caused by serotype 19A was more diverse (Table 3). Among episodes resulting in death, 3 were caused by non-PCV7 serotypes (serotypes 7F, 27, and 6A) and 1 by vaccine serotype 23F in an unvaccinated child.

PCV7 serotypes were significantly present in younger children (mean age, 21.2 vs. 30.4 months in IPD caused by non-PCV7 serotypes; $P = 0.004$). In addition, IPD by PCV7 serotypes was associated with a higher rate of sequelae than non-PCV7 serotypes (25.9% vs. 9.9%; $P = 0.02$). In contrast, non-PCV7 serotypes were associated mainly with pneumonia: 81.3% of total episodes caused by non-PCV7 versus 48.1% of episodes caused by PCV7 serotypes; $P < 0.001$ (Table 3).

TABLE 2. Characteristics of Vaccinated Children With Invasive Pneumococcal Disease Caused by PCV7 Serotypes

Sex	Age (mo)	Clinical Presentation	Previous Disease	Serotype
Female	5	Bacteremia	Methylmalonic acidosis	19F
Female	13	Bacteremia	Retinoblastoma (neutropenia)	19F
Female	45	Pneumonia	No	19F
Male	29	Pneumonia	No	14
Female	50	Pneumonia	No	14

Molecular analysis by MLST was performed for 108 of 123 (87.8%) strains isolated by culture. Overall, when comparing our data with isolates listed in the MLST database, there were 46 different STs, including 8 new ST profiles (ST3437, serotype 23F; ST3436, serotype 38; ST4827, ST2948, and ST4826, serotype 19F; ST4676, serotype 27; ST5195, serotype 19A; ST4834 serotype 7F). Of these, 50% new ST expressed PCV7 serotypes. eBURST analysis using the stringent 6/7 identical loci definition grouped the 46 ST into 6 CCs and 34 singletons (Fig. Supplemental Digital Content 2, http://links.lww.com/INF/B15, shows clonal distribution of 108 invasive isolates from pediatric patients obtained by use of the output of eBURST, version 3. Each circle represents single MLST, with the area proportional to the number of isolates of that ST. Black lines represent single-locus variants).

Six CCs or ST accounted for 55.9% of total collection: ST306 (n = 22 isolates serotype 1), ST320 (n = 9 isolates serotype 19A), CC289 (n = 8 isolates serotype 5), ST191 (n = 8 isolates serotype 7F), ST1201 (n = 7 isolates serotype 19A), and CC276 (n = 5 isolates serotype 19A and 1 serotype 24B).

Comparative analysis of our serotype and ST results with those published in the MLST database showed that 5 of our STs expressed serotypes different than those previously reported (capsular switching): ST101 (serotype 15C), ST109 (serotype 23F), ST230 (serotype 24B), ST433 (serotype 28), and ST2372 (serotype 23F). Antibiotic susceptibility was available for 120 of 123 (97.5%) strains.

None of the 120 strains was fully resistant (MIC >8 μg/mL) and 3 (2.5%) were intermediately penicillin-resistant according to nonmeningeal breakpoints. Two of these strains be-

TABLE 3. Epidemiologic Data and Clinical Characteristic of 300 Episodes of IPD Caused by PCV7 Serotypes, Non-PCV7 Serotypes, and the 5 Main Serotypes Detected in the Study

Serotype	No. Episodes	Age (Mean SD)	Sex (Males) N (%)	Clinical Presentation*				PICU Admission N (%)	Outcome	
				Pneumonia N (%)	Bacteremia N (%)	Meningitis N (%)	Others N (%)		Sequelae	Death
PCV7	27	21.2 (13.68)	16 (59.3%)	13 (48.1%)	7 (25.9%)	6 (22.2%)	1 (3.7%)	6 (22.2%)	7 (25.9%)	1 (3.7%)
Non–PCV7	273	30.4 (15.84)	145 (53.1%)	222 (81.3%)	18 (6.6%)	23 (8.4%)	10 (3.6%)	38 (13.9%)	27 (9.9%)	3 (1.1%)
Serotype 1	62	41 (10.48)	34 (54.8%)	62 (100%)	0	0	0	2 (3.2%)	3 (4.8%)	0
Serotype 19A	47	19.21 (10.54)	28 (59.6%)	32 (68.1%)	6 (12.8%)	6 (12.8%)	3 (6.3%)	9 (19.1%)	4 (8.5%)	3 (2.4%)
Serotype 3	37	29.03 (14.11)	16 (43.2%)	36 (97.3%)	0 (0%)	1 (2.7%)	0	6 (16.2%)	5 (13.5%)	0
Serotype 7FA	21	24.71 (16.2)	16 (76.2%)	12 (57.1%)	4 (19%)	4 (19%)	1 (4.9%)	5 (23.8%)	4 (19%)	1 (4.8%)
Serotype 14	12	24.71 (13.3)	9 (75%)	11 (91.7%)	1 (8.3%)	0	0	0	3 (25%)	0

*Other clinical presentations were arthritis or osteomyelitis, sepsis, and cellulitis.

Statistically significant differences (χ^2 test for categorical variables and Student t test for continuous variables) were found for the following.

Mean age: PCV7 versus non-PCV7 serotypes ($P = 0.004$); serotype 1 versus other serotypes ($P < 0.001$); serotype 19A versus other serotypes ($P < 0.001$).

Gender: serotype 7F/A versus other serotypes ($P = 0.03$).

Clinical presentation: serotype 1 versus other serotypes ($P = 0.001$).

PICU admission: serotype 1 versus other serotypes ($P = 0.002$).

Outcomes: PCV7 versus non-PCV7 serotypes ($P = 0.02$).

longed to ST320 expressing serotype 19A and the other belonged to ST2948 expressing serotype 19F. Forty-one isolates (34, 4%) had an MIC $\geq$0.12 μg/mL, and 18 of these isolates (43.9%) were serotype 19A. Regarding cefotaxime, only 2 isolates (1.7%) showed an MIC $\geq$4, and both belonged to ST320-expressing serotype 19A. Regarding meningeal breakpoints, 21 isolates (17.5%) showed a diminished susceptibility to cefotaxime, and serotype 19A account 66.7% of these episodes.

DISCUSSION

This is a prospective study that updates the information about IPD in children in a geographical area without systematic vaccination. The inclusion of episodes with negative culture and only detected by real-time PCR has allowed us to gain greater insight into the burden of the disease and the main serotypes causing IPD in Barcelona. We think that molecular methods can be used directly not only on samples as cerebrospinal fluid, pleural effusion, or arthritis fluid but also in plasma improving the ability to diagnose IPD. The usefulness of real-time PCR in blood has been discussed because some authors found a high rate of detection of pneumococcal DNA in healthy controls associated with nasopharyngeal carriage.[20] However, we consider plasma PCR–positive patients with noncomplicated pneumonia and negative culture as patients with pneumococcus pneumonia and not false positive from pneumococcus colonization. All these patients are clinically compatible with pneumococcus pneumonia (all of them have high fever, cough, crackling, or hypophonesis in the auscultation and radiologic image of alveolar condensation). Moreover, our patients with noncomplicated pneumonia have no statically differences in clinical variables in the groups "positive blood culture" and "only plasma real-time PCR positive." In the same way, other authors have described previously the validity of plasma PCR in diagnosing IPD.[8,11] A low bacterial load could explain the negativity of the culture in these patients. However, more studies in this area will be required to confirm the validity of plasma PCR in determining deep-seated pneumococcal infection.

The incidence of IPD continues to increase in our geographic area. The incidence is higher than previously reported, presumably as a result of low sensitivity of the bacterial culture, which was the only microbiologic criterion for definition of IPD in previous studies.[5] The hospitals included in the study are the most important ones in pediatrics in Catalonia. Non-PCV7 serotypes cause most IPD episodes, whereas PCV7 serotypes cause only a minority of cases. The change in pneumococcal serotypes causing IPD is associated with a change in clinical presentation and in some epidemiologic characteristics.

Concerning clinical manifestations, the proportion of pneumococcal bacteremia and meningitis is relatively stable, but a significant increase in pneumonia was observed. These changes were observed by others in the United States.[21]The increase in pneumonia has also been observed in other regions of Spain[22] and in other countries such as Denmark and United States.[23,24] A significant proportion of these pneumonias is complicated by empyema, and some of the children (5.5%) developed pulmonary sequelae and required intensive care management. This high proportion of empyema and parapneumonic pleural effusion could also be explained because the study was performed in 2 tertiary-care pediatric hospitals. Of concern is the increase in complicated pneumonias caused by non-PCV7 serotypes. It is important that vaccines against *S. pneumoniae* include serotypes associated with pneumonia, such as serotypes 1 and 3.

The mean age of children with IPD is higher than previously reported in the prevaccine era,[25] and the majority of children with IPD is healthy without any recognized risk factors. This high proportion of healthy children is different from what was recently reported by Kaplan et al.[21] These differences are caused in part by the introduction of a virulent clone of serotype 1, with proven capacity to produce outbreaks, just before the implementation of PCV7 in our country.[5] Serotype 1, which is associated with pneumonia in older healthy children,[22] was the main serotype detected in our series, whereas in Kaplan et al's study, serotype 1 was infrequent.

It is remarkable how many serotypes are involved, which demonstrates the great diversity of pneumococcus. The detection of only 24 serotypes by multiplex real-time PCR methodology is a limitation of the study and raises concern about the high diversity of pneumococcus and the need for accurate surveillance of this disease in the coming years, including new molecular methods to detect a wider range of serotypes.

Despite the low vaccination coverage (approximately 50%), a low rate of infections due to vaccine serotypes were found. These data confirm, as have many other studies, that PCV7 is highly effective against IPD caused by vaccine serotypes, because this vaccine also prevents IPD in adult contacts and nonvaccinated siblings through indirect effect (herd immunity) on pneumococcal transmission.[26]

Regarding the 5 cases of IPD in vaccinated children, it is important to note that 2 of them had a previous disease and had not completed the vaccination schedule, which might explain this failure.

As to the clonal study, it also showed great genetic diversity in the strains that produce IPD in our pediatric population, including the appearance of new ST and capsular switches.

As reported before,[5] ST306 is the most important ST in our population. This ST relates to an increase of empyema.[22] Recently, our group showed that this increase of empyema associated with ST306 may be because of the presence of PsrP,[27] a pneumococcal virulence factor not present in all STs of *S. pneumoniae*. PsrP is an adhesine related to the invasion of pulmonary cells by pneumococcus.

As for the study of antibiotic susceptibility, we previously reported a decrease in the global rate of penicillin resistance if we compare the present rate with that of the prevaccine period.[5] Nevertheless, the presence of strains of serotype 19A, especially those with ST320 having multiple antibiotic resistances, is grounds for concern. PCV13 includes serotype 19A, which we hope will be controlled once the new vaccine is implemented.

One of the limitations of the study is that not all episodes of IPD are detected, as blood cultures and/or PCR *S. pneumoniae* are not performed in all children with fever or suspected of pneumonia. Therefore, some bacteremias and pneumonias have presumably not been diagnosed. However, our guidelines for evaluating children with fever did not substantially change during the study period.

In conclusion, IPD continues to increase in Barcelona, and the rate is much higher than previously reported due to low sensitivity of bacterial culture. Non-PCV7 serotypes were responsible for 91% of episodes, and pneumonia was the main clinical presentation.

REFERENCES

1. O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet*. 2009;374:893–902.
2. Hausdorff WP, Bryant J, Paradiso PR, et al. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part 1. *Clin Infect Dis*. 2000;30:100–121.
3. Black S, Shinefield H, Baxter R, et al. Postlicensure surveillance for pneumococcal disease after use of heptavalent pneumococcal conjugate vaccine in Northern California Kaiser Permanente. *Pediatr Infect Dis J*. 2004;23:485–489.
4. Whitney CG, Farley MM, Hadler J, et al. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med*. 2003;348:1737–1746.
5. Muñoz-Almagro C, Jordan I, Gene A, et al. Emergence of invasive pneumococcal disease caused by non-vaccine serotypes in the era of 7-valent conjugate vaccine. *Clin Infect Dis*. 2008;46:174–182.
6. Hausdorff WP, Bryant J, Kloek C, et al. The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II. *Clin Infect Dis*. 2000; 30:122–140.
7. Muñoz-Almagro C, Gala S, et al. DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema. *Eur J Clin Microbiol Infect Dis*. 2011;30:327–335.
8. Azzari C, Moriondo M, Indolfi G, et al. Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. *J Med Microbiol*. 2008;57:1205–1212.
9. Corless CE, Guiver M, Borrow R, et al. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. *J Clin Microbiol*. 2001;39:1553–1558.
10. Van Gastel E, Bruynseels P, Verstrepen W, et al. Evaluation of a real-time polymerase chain reaction assay for the diagnosis of pneumococcal and meningococcal meningitis in a tertiary care hospital. *Eur J Clin Microbiol Infect Dis*. 2007;26:651–653.
11. Lahti E, Mertsola J, Kontiokari T, et al. Pneumolysin polymerase chain reaction for diagnosis of pneumococcal pneumonia and empyema in children. *Eur J Clin Microbiol Infect Dis*. 2006;25:783–789.
12. Picazo J, Ruiz-Contreras J, Casado-Flores J, et al. Relationship between serotypes, age, and clinical presentation of invasive pneumococcal disease in Madrid, Spain, after introduction of the 7-valent pneumococcal conjugate vaccine into the vaccination calendar. *Clin Vaccine Immunol*. 2011;18:89–94.
13. Web de l'Estadística Oficial de Catalunya. Institut d'Estadística de Catalunya. Available at: http://www.idescat.net. Accessed August 11, 2010.
14. National Committee for Clinical Laboratory Standards (NCCLS). *Performance Standards for Antimicrobial Susceptibility Testing (2008): Eighteenth Informational Supplement.* Wayne, PA: Clinical and Laboratory Standard Institute; 2008. CLSI document M100-S18 (ISBN 1–5–56238–653–0).
15. Fenoll A, Jado I, Vicioso D, et al. Dot blot assay for the serotyping of pneumococci. *J Clin Microbiol*. 1997;35:764–766.
16. Tarragó D, Fenoll A, Sánchez-Tatay D, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. *Clin Microbiol Infect*. 2008;14:828–834.
17. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology*. 1998;144:3049–3060.
18. Feil EJ, Li BC, Aanensen DM, et al. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol*. 2004;186:1518–1530.
19. American Academy of Pediatrics, Committee on Infectious Diseases. Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis. *Pediatrics*. 2000;106:362–366.
20. Dagan R, Shriker O, Hazan I, et al. Prospective study to determine clinical relevance of detection of pneumococcal DNA in sera of children by PCR. *J Clin Microbiol*. 1998;36:669–673.
21. Kaplan SL, Barson WJ, Lin PL, et al. Serotype 19A is the most common serotype causing invasive pneumococcal infections in children. *Pediatrics*. 2010;125:429–436.
22. Obando I, Muñoz-Almagro C, Arroyo LA, et al. Pediatric parapneumonic empyema, Spain. *Emerg Infect Dis*. 2008;14:1390–1397.
23. Winther TN, Kristensen TD, Kaltoft MS, et al. Invasive pneumococcal disease in Danish children, 1996–2007, prior to the introduction of heptavalent pneumococcal conjugate vaccine. *Acta Pediatr*. 2009;98:328–331.
24. Byington CL, Korgenski K, Daly J, et al. Impact of the pneumococcal conjugate vaccine on pneumococcal parapneumonic empyema. *Pediatr Infect Dis J*. 2006;25:250–254.
25. Robinson K, Baughman W, Rothrock G, et al. Epidemiology of *Streptococcus pneumoniae* infections in the U.S., 1995–1998: opportunities for prevention in the conjugate vaccine era. *JAMA*. 2001;285:1729–1735.
26. Centers for Disease Control and Prevention (CDC). Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease—United States, 1998–2003. *Morb Mortal Wkly Rep*. 2005;54:893–897.
27. Muñoz-Almagro C, Selva L, Sanchez CJ, et al. PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia and is strongly associated with clonal type. *Clin Vaccine Immunol*. 2010;17:1672–1678.

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Effectiveness of 7-valent pneumococcal conjugate vaccine in the prevention of invasive pneumococcal disease in children aged 7–59 months. A matched case-control study

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ABSTRACT

The aim of this study was to evaluate the effectiveness of the administration of the 7-valent pneumococcal conjugate vaccine in a region with an intermediate vaccination coverage.

A matched case-control study was carried out in children aged 7–59 months with invasive pneumococcal disease (IPD) admitted to two university hospitals in Catalonia. Three controls matched for hospital, age, sex, date of hospitalization and underlying disease were selected for each case. Information on the vaccination status of cases and controls was obtained from the vaccination card, the child's health card, the hospital medical record or the vaccination register of the primary healthcare center where the child was attended for non-severe conditions. A conditional logistic regression analysis was made to control for the effect of possible confounding variables.

The adjusted vaccination effectiveness of the complete vaccination schedule (3 doses at 2, 4 and 6 months and a fourth dose at 15 months, 2 doses at least two months apart in children aged 12–23 months or a single dose in children aged >24 months) in preventing IPD caused by vaccine serotypes was 93.7% (95% CI 51.8–99.2). It was not effective in preventing cases caused by non-vaccine serotypes.

The results of this study carried out in a population with intermediate vaccination coverage confirm those of other observational studies showing high levels of effectiveness of routine 7-valent pneumococcal conjugate vaccination.

1. Introduction

The importance of pneumococcal infection in children aged <2 years [1–3] and growing pneumococcal resistance to antibiotics [4] stimulated the search for conjugated pneumococcal vaccines, of which the first, the 7-valent vaccine (PCV7) was licensed in 2000 in the United States, after a controlled clinical trial demonstrated protective efficacy against invasive pneumococcal disease (IPD) in small children [5,6]. The vaccine was licensed in Spain in 2001 [2].

In the 1990s, the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) were mainly responsible for cases of IPD in children in the United States [7] and 71% of cases of IPD in children aged <2 years in Catalonia [8], a region in Northeastern Spain with more than 7 million inhabitants.

In Spain, all routine vaccines included in the official vaccination calendar are administered free of charge. Except for the Madrid region, the PCV7 has not been included in the official vaccination calendar, but is recommended by the Spanish Association of Pediatrics [9]. In Catalonia (Spain), many private and public pediatric practices recommend the vaccine, which is paid for by parents. The recommended schedule is three doses at 2, 4 and 6 months, with a fourth dose at 15 months. Two doses at least 2 months apart are recommended in children aged 12–23 months and a single dose in children aged ≥2 years. A 2004 Catalan study found a vaccination

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coverage of 27% [10] and later studies found coverages of 34% and 50% [11,12].

This vaccination strategy has achieved only intermediate vaccination coverages and has had a limited impact [13]. Unlike the Unites States [14], in Catalonia the global incidence of IPD has hardly diminished after licensing of the vaccine [13]. The overall incidence of IPD in Catalonia in children aged < 2 years was 53.7 per 100,000 persons-year in 2001 and 56.4 in 2008 [15]. The proportion of vaccine serotypes causing IPD has diminished due to vaccination, but the proportion of non-vaccine serotypes has increased, explaining the limited impact [11,12,16–19]. Some authors [20–24] have shown that the PCV7 is effective in preventing IPD caused by vaccine serotypes, but it is important to investigate the effectiveness of the vaccine in different age groups in communities where vaccination coverages are not very high.

The purpose of this study was to evaluate the effectiveness of the PCV7 (protective value of vaccination in usual circumstances in the field) in children aged 7–59 months in a population with intermediate vaccination coverages.

2. Methods

2.1. Study design

A matched case-control study was carried out in patients with IPD admitted to two university hospitals in Barcelona (Hospital Sant Joan de Déu and Hospital Vall d' Hebron) with 339 and 210 pediatric beds, respectively. There are about 400,000 children aged <5 years in Catalonia and the two study hospitals attend more than 30% of this population. Both hospitals form part of the Network of Public Hospitals of Catalonia and all care are free at the point of use. All children from the assigned population requiring hospitalization are referred by private or public health pediatricians to these hospitals. Therefore, the study population can be considered as representative of the population aged <5 years in Catalonia. The study period was January 1, 2007 to December 31, 2009.

2.2. Case selection

All patients aged 7–59 months hospitalized for IPD were initially studied. Children aged 0–6 months were excluded as they had not had the opportunity to receive primary immunization according to the schedule recommended in the technical data sheet, i.e., three doses of vaccine administered during the six first months of age. IPD was defined as isolation of *Streptococcus pneumoniae* or detection of DNA of the pneumolysin (*ply*) gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in any normally sterile site. Children who presented IPD in whom serotyping of *S. pneumoniae* was not possible because the sample was not available were excluded.

Cases of IPD were clinically classified as: pneumonia, pneumonia complicated by empyema, meningitis, occult bacteremia or sepsis and other clinical forms. Pneumonia and pneumonia with empyema were diagnosed clinically and radiologically (pneumonia alone or pneumonia plus pleural effusion).

Strains of *S. pneumoniae* isolated by culture were identified by identical standard microbiological methods throughout the study period. Detection of the *ply* gene of *S. pneumoniae* was performed by real-time PCR according to a previously reported method [25].

Strains isolated by culture were serotyped using the Quellung reaction or dot blot by the National Centre for Microbiology, Majadahonda, Madrid. Serotypes in patients with negative cultures were detected at the Sant Joan de Déu Hospital by multiplex real-time PCR [26], which detects the conserved *wzg* capsule gene and other genes selected to differentiate the 24 serotypes most frequently implicated in IPD (1, 3, 4, 5, 6A, 6B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A and 23F). Serotypes were classified into 2 groups: vaccine serotypes (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F), and non-vaccine serotypes (all others). Since PCR does not differentiate between serotypes 9V, 9A, 9N and 9L, between serotypes 18C and 18B or between serotypes 19F, 19B and 19C these serotypes were all considered as vaccine serotypes 9V, 18C or 19F, respectively.

2.3. Selection of controls

Three controls for each case were selected from patients aged 7–59 months treated in the same hospitals. Controls were selected prospectively in wards and outpatient visits for non-infectious diseases and matched by age (±3 months if aged 7–11 months, but always older than 6 months, and ± 6 months if aged 12–59 months), sex, date of hospitalization or outpatient visit at the same center (±30 days) and underlying risk condition when present. Risk conditions are detailed in the clinical variables section.

Study investigators were blinded to the vaccination status of all controls during selection.

2.4. Vaccination status of cases and controls

A case was considered vaccinated if they had received the last dose (or only dose if this was the schedule corresponding to their age) of the PCV7 ≥ 15 days before symptom onset. Controls were considered vaccinated if they had received the last or only dose ≥15 days before hospital admission or outpatient visit. A child was considered completely vaccinated when they had received the recommended doses corresponding to age. A case or control was considered vaccinated when confirmed by records. For both cases and controls, the vaccination card or health card, where pediatricians (both public and private) record the vaccines administered and the date, were asked for. If neither was available, hospital medical records were consulted and, finally, if there was no record of vaccination, registers of the primary healthcare center where the child was attended for non-severe conditions were consulted.

The number of doses received and the date of administration were recorded. Cases and controls whose vaccination status could not be determined were excluded.

2.5. Sociodemographic, clinical and epidemiological variables

The demographic and clinical variables recorded for each case were: age, sex, date of hospitalization, clinical form of IPD, risk medical conditions [27] (sickle cell disease, congenital or acquired asplenia, HIV infection, cochlear implants, congenital immune deficiency, chronic heart disease, chronic pulmonary disease including asthma if treated with high risk-dose oral corticosteroid therapy, cerebrospinal fluid leaks, chronic renal failure including nephrotic syndrome, immunosuppressive or radiation therapy, solid organ transplantation and diabetes mellitus), and antibiotic therapy and history of respiratory infection in the 30 days before symptom onset. Other epidemiological variables recorded were: day care or school attendance, parental smoking, number of cohabitants, age of siblings and parental socioeconomic level, classified into six groups according to parental occupation using the British Classification of Occupations [28]. Two levels were considered: high (classes I–III) and low (classes IV–V).

The same variables were recorded for controls except for those relating to IPD. All variables were collected using a single questionnaire for cases and controls and there was an instruction manual to facilitate compliance.

Table 1
Distribution of serotypes causing invasive pneumococcal disease according to age.

	7–23 Months		24–59 Months		7–59 Months	
	No.	%	No.	%	No.	%
Vaccine serotypes	14	15.1%	10	5.6%	24	8.9%
9V	0	0%	1	0.6%	1	0.4%
14	6	6.5%	6	3.4%	12	4.4%
18C	1	1.1%	0	0%	1	0.4%
19F	5	5.4%	2	1.1%	7	2.6%
23F	2	2.2%	1	0.6%	3	1.1%
Non-vaccine serotypes	79	84.9%	168	94.4%	247	91.1%
1	5	5.4%	57	32.0%	62	22.9%
3	16	17.2%	21	11.8%	37	13.7%
5	0	0%	7	3.9%	7	2.6%
6A	3	3.2%	1	0.6%	4	1.5%
7F	6	6.5%	10	5.6%	16	5.9%
10A	1	1.1%	0	0%	1	0.4%
15A	1	1.1%	1	0.6%	2	0.7%
19A	27	29.0%	14	7.9%	41	15.1%
22F	0	0%	1	0.6%	1	0.4%
23B	2	2.2%	0	0%	2	0.7%
24	1	1.1%	0	0%	1	0.4%
24F	1	1.1%	0	0%	1	0.4%
38	1	1.1%	0	0%	1	0.4%
Non-typeable	15	16.1%	56	31.5%	71	26.2%
Total	93	34.3%	178	65.7%	271	100%

2.6. Sample size

The sample size required was calculated using Schlesselman's criteria [29]. Assuming a prevalence of history of vaccination in controls of 25% (data from Catalonia before the start of the study), a vaccination effectiveness of 80%, a bilateral α error of 0.05 (two-tailed), a β error of 0.2, and supposing that 20% of cases would be caused by vaccine serotypes (preliminary data), that three controls would be sought per case, and that children aged <2 years and those aged 24–59 months would be analyzed separately, the minimum number of cases required was estimated at 270 and the number of controls as 810.

2.7. Statistical analysis

Differences in demographic and epidemiological variables between cases and controls were analyzed using Pearson's chi-square test for categorical variables and the Student *t*-test for continuous variables. A two-tailed distribution was assumed for all *p*-values. The crude odds ratio (OR) and their 95% confidence intervals (CI) taking into account the distribution of completely vaccinated children and unvaccinated children and IPD were estimated using McNemar's chi-square test.

To avoid the effect of possible confounding variables, multivariate analysis was performed using conditional logistic regression and including independent variables found to be associated with both the disease and vaccination with a cut-off point of $p < 0.1$ in the bivariate analysis. The variable age was also included due to its relevance.

Vaccination effectiveness (VE) was calculated using the formula $VE = (1 - OR) \times 100$. Analyses were performed for vaccine and non-vaccine serotypes and age group. The statistical power of the analyses made was calculated using Schlesselman's formula [29].

The analysis was performed using the SPSS v18 statistical package.

2.8. Data confidentiality and ethical aspects

All data was treated as confidential in accordance with legislation on observational studies. Because there was no intervention, and the health care provided to cases and controls was the same whether parents agreed to participate or not, cases and controls were enrolled if a parent or guardian provided oral informed consent. The Institute of Health Studies, Generalitat of Catalonia and the Ethics Committee, Fundació Hospital Sant Joan de Déu approved the study.

3. Results

During the study period, 293 cases of IPD were detected in patients aged 7–59 months, of which 271 were included. The remaining cases were excluded because the vaccination history was not determined (2 cases), no controls meeting the study criteria were found (1 case) or the sample for serotyping was not available (19 cases).

Of the 271 cases, 98 (36.2%) were diagnosed by culture and real-time PCR and 173 (63.8%) by real-time PCR only. 8.9% were caused by vaccine serotypes and 91.1% by non-vaccine serotypes (Table 1). The most-frequent serotypes were 1 (22.9%), 19A (15.1%) and 3 (13.7%). No changes occurred in the real-time PCR methodology during the study period.

A total of 65.7% of cases were aged 24–59 months and 34.3% were aged <24 months (Table 1). The most-frequent serotypes identified were 1 (32.0%), 3 (11.8%) and 19A (7.9%) in the 24–59 months age group and 19A (29.0%), 3 (17.2%) and 14 (6.5%) in the 7–23 months age group.

The most frequent clinical forms were pneumonia with empyema (59% of cases), pneumonia without empyema (26.2%), meningitis (7.4%) and non-focal bacteremia or sepsis (5.9%) (Table 2). Other clinical forms were cellulitis (2 cases) and osteoarticular infection (2 cases). Pneumonia with empyema increased from 37 cases in 2007 to 65 in 2009. No increase was observed in the remaining clinical forms. The most-frequent serotypes were 1 (47 cases), 3 (28 cases) and 19A (27 cases) in cases of pneumonia with empyema and serotype1 (15 cases) and serotype 3 (8 cases) in cases of pneumonia without empyema. In cases of meningitis and non-focal bacteremia or sepsis the most frequent serotype was 19A (3 cases and 4 cases, respectively).

Of the 751 possible controls aged 7–59 months selected, 747 were finally included. The remaining 4 children were excluded because the vaccination history was not determined. Cases presented similar characteristics to controls, with the exception of

Table 2
Distribution of invasive pneumococcal disease-causing serotypes according to age and clinical form.

	Pneumonia without empyema		Pneumonia with empyema		Meningitis		Non-focal bacteremia/sepsis	
	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)	7–23 months No. (%)	24–59 months No. (%)
Vaccine serotypes	2 (10.0%)	6 (11.8%)	3 (7.5%)	2 (1.7%)	4 (25.0%)	1 (25.0%)	5 (38.5%)	1 (33.3%)
9V	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
14	2 (10.0%)	4 (7.8%)	3 (7.5%)	2 (1.7%)	0 (0.0%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
18C	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
19F	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	1 (25.0%)	4 (30.8%)	0 (0.0%)
23F	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (12.5%)	0 (0.0%)	0 (0.0%)	1 (33.3%)
Non-vaccine serotypes	18 (90.0%)	45 (88.2%)	37 (92.5%)	118 (98.3%)	12(75.0%)	3 (75.0%)	8 (61.5%)	2 (66.7%)
1	1 (5.0%)	14 (27.4%)	4 (10.0%)	43 (35.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
3	6 (30.0%)	2 (3.9%)	10 (25.0%)	18 (15.0%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
5	0 (0.0%)	2 (3.9%)	0 (0.0%)	4 (3.3%)	0 (0.0%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
6A	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
7F	1 (5.0%)	4 (7.8%)	2 (5.0%)	5 (4.1%)	2 (12.5%)	0 (0.0%)	1 (7.7%)	1 (33.3%)
10A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
15A	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	1 (25.0%)	0 (0.0%)	0 (0.0%)
19A	4 (20.0%)	1 (2.0%)	14 (35.0%)	13 (10.8%)	3 (18.7%)	0 (0.0%)	4 (30.8%)	0 (0.0%)
22F	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (33.3%)
23B	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
24	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
24F	0 (0.0%)	0 (0.0%)	1 (2.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Non-typeable	6 (30.0%)	21 (41.2%)	6 (15.0%)	35 (29.2%)	2 (12.5%)	0 (0.0%)	1 (7.7%)	0 (0.0%)
Total	20 (7.5%)	51 (19.1%)	40 (15.0%)	120 (44.9%)	16 (6.0%)	4 (1.5%)	13 (4.9%)	3 (1.1%)

greater day care or school attendance in cases compared with controls ($p < 0.01$) and more cohabitants in cases than in controls ($p = 0.003$). Complete vaccination was lower in cases aged 7–23 months than in children aged 24–59 months (41.9% and 47.8%, respectively; $p = 0.035$) (Table 3).

After adjusting for age, vaccination effectiveness (complete vaccination schedule) was 93.7% (95% CI 51.8–99.2) for vaccine serotypes. There was no protection against IPD caused by non-vaccine serotypes (Table 4).

Vaccination effectiveness was higher in the 7–23 months age group than in the whole study group. In the 24–59 months age group, no statistically significant differences were found (Table 4).

4. Discussion

As in most countries [30], only a few of the 93 known *S. pneumoniae* serotypes cause IPD in children aged <5 years in Catalonia. In our study, 67.3% of all cases were caused by 7 serotypes (14, 19 F, 19 A, 1, 3, 5, and 7F) (Table 1). However, after the PCV7 was marketed in Catalonia, the proportion of cases caused by PCV 7serotypes has fallen substantially, from 76.2% in 1999 [8] to 8.9% during the study period.

Although only 47% of controls were completely vaccinated, the marked reduction found in cases of IPD due to vaccine serotypes suggests that herd protection has played some role in Catalonia.

The predominant clinical form was pneumonia with empyema which is mostly caused by non-vaccine serotypes and is increasingly frequent in Spain, with Calbo et al. [11] finding that the rate rose from 1.7 per 100,000 in the prevaccination era (1999–2001) to 8.5 in the postvaccination era (2002–2004), a five-fold increase, and Muñoz-Almagro et al. [12] finding that cases rose seven-fold between 1997–2001 and 2002–2006. Our results show that, in the 36-month study period, cases of pneumonia with empyema rose from 37 in 2007 to 65 in 2009, a phenomenon that has occurred to a lesser degree in the United States [31], but not in England [32]. Because no changes in diagnostic methods occurred during the study period, this increase can be considered as real and not a consequence of more diagnoses made by PCR.

The fact that three doses of PCV7 in infants aged <1-year old were required to consider a case or control as completely

Table 3
Characteristics of cases and controls.

Characteristic	Cases $N = 271$	Controls $N = 747$	p-Value
Months, median (range)	33.0 (7–59)	31.0 (7–59)	0.69*
Gender			0.57†
Male	145 (53.5%)	414 (55.5%)	
Female	126 (46.5%)	332 (44.5%)	
Period of hospitalization			0.16†
January–March	86 (31.7%)	263 (35.3%)	
April–June	55 (20.3%)	180 (24.1%)	
July–September	21 (7.7%)	59 (7.9%)	
October–December	109 (40.3%)	244 (32.7%)	
Underlying disease			0.81†
Yes	3 (1.1%)	7 (0.9%)	
No	268 (98.9%)	739 (99.1%)	
Social class			
I– III	143 (59.6%)	333 (57.7%)	0.62†
IV–V	97 (40.4%)	244 (42.3%)	
Attendance at day care or school			<0.01†
Yes	226 (86.3%)	550 (73.8%)	
No	36 (13.7%)	195 (26.2%)	
Antibiotic treatment in previous month			0.11†
Yes	41 (15.5%)	148 (19.9%)	
No	224 (84.5%)	596 (80.1%)	
Infection in previous month			0.71†
Yes	127 (48.1%)	348 (46.8%)	
No	137 (51.9%)	396 (53.2%)	
Breastfeeding			0.28†
Yes	197 (75.5%)	523 (72.0%)	
No	64 (24.5%)	203 (28.0%)	
Exposure to tobacco in the home			0.32†
Yes	106 (40.6%)	313 (44.1%)	
No	155 (59.4%)	396 (55.9%)	
Cohabitants, mean (range)	4.02 (2–10)	3.81 (2–9)	0.003*
Siblings			0.29†
Yes	139 (53.3%)	421 (57.0%)	
No	122 (46.7%)	317 (43.0%)	
Siblings < 5 years of age			0.12†
Yes	76 (29.1%)	191 (25.9%)	
No	185 (70.9%)	547 (74.1%)	
Complete vaccination			
7–23 months	39 (41.9%)	150 (55.1%)	0.03†
24–59 months	85 (47.8%)	201 (42.3%)	0.22†
7–59 months	124 (45.8%)	351 (47.0%)	0.71†

* Student *t*-test.
† Pearson's chi-square test.

Table 4
Adjusted effectiveness of the 7-valent pneumococcal conjugate vaccination in the prevention of invasive pneumococcal disease in completely vaccinated children aged 7–59 months.[a]

Serotype	Cases	Controls	Crude vaccination effectiveness			Adjusted vaccination effectiveness[b]			
	vaccinated/N (%)	vaccinated/N (%)	%	95% CI	*p* value	%	95% CI	*p* value	Power
All serotypes	124/251 (49.4%)	351/658 (53.3%)	16.1%	−13.5–37.9	0.25	13.2%	−20.7–37.6	0.40	12.6%
Vaccine serotypes									
7–23 Months	1/14 (7.1%)	24/40 (60.0%)	92.3%	38.1–99.0	0.02	92.5%	39.3–99.1	0.02	98.5%
24–59 Months	3/9 (33.3%)	12/21 (57.1%)	79.2%	−84.7–97.7	0.16	79.4%	−84.0–97.7	0.16	40.5%
7–59 Months	4/23 (17.4%)	36/61 (59.0%)	93.8%	51.9–99.2	0.01	93.7%	51.8–99.2	0.01	99.8%
Non-vaccine serotypes	120/228 (52.6%)	315/597 (52.8%)	−8.0%	−56.4–25.4	0.68	−10.9%	−65.6–25.7	0.61	18.9%

[a] Incomplete vaccinated children were excluded from the analysis.
[b] Adjusted using conditional logistic regression for attendance at day care or school (all serotypes and non-vaccine serotypes), cohabitants (all serotypes and non-vaccine serotypes) and age (all serotypes, vaccine serotypes in all age groups and non-vaccine serotypes).

vaccinated is important, because the benefits of this schedule against a reduced-dose regimen have been reported recently [33].

The adjusted effectiveness of the complete vaccination schedule was high against IPD caused by vaccine serotypes (93.7%, 95% CI 51.8–99.2) and was not effective against non-vaccine serotypes (−10.9%, 95% CI −65.6–25.7).

The point effectiveness of the complete vaccination schedule against cases caused by vaccine serotypes was similar to that observed in case-control studies by Whitney et al. [20] (96%; 95% CI 93–98), Deceuninck et al. [21] (92%; 95% CI 83–96), and Barricarte et al. [22] (88%; 95% CI 9–98), and in indirect cohort studies by Mahon et al. [23] (90.5%; 95% CI 17.7–98.9), and Rückinger et al. [24] (94.6%; 95% CI 69.7–99.5). The confidence intervals of these studies overlap with ours.

The study of effectiveness according to age showed that, in children aged 7–23 months, the vaccine was very effective in preventing cases due to vaccine serotypes when the complete vaccination schedule was administered (92.5%; 95% CI 39.3–99.1). In the 24–59 months age group, there was a non-significant trend to protection (79.4%; 95% CI-84.0–97.7). However, the statistical power of the study was only 40.5% in this age group due to the small sample size (only 10 cases of IPD due to vaccine serotypes). The fact that a lower proportion of cases due to vaccine serotypes was found in children aged > 24 months is probably related to the higher vaccine coverage found in this age group with respect to the 7–23 months age group (47.8% and 41.9, respectively). Another possible reason is the higher proportion of cases due to serotype1 reported in children aged > 24 months in Catalonia [34].

The small number of vaccine serotype cases included in the study did not confer enough power to analyze vaccination effectiveness according to clinical form, number of doses received, single serotype or risk medical conditions.

The PCV7 reduces the prevalence of carriers of vaccine serotypes and, possibly, of serotype 6A [35]. Since nasopharyngeal carriers of *S. pneumoniae* are the main source of IPD, this reduction results in herd immunity, indirectly protecting unvaccinated people. In case-control studies matched by hospital and date of admission, such as our study, patients and controls probably have the same probability of benefiting from indirect protection. In fact, this type of study primarily measures direct protection, and other designs are necessary to estimate indirect protection [36]. The Centers for Disease Control and Prevention made this estimate in the United States using epidemiological disease surveillance and concluded that indirect protection prevented more cases (20,459) than direct protection (9149) in the four years after introduction of the vaccine [37].

Our study, like all observational studies, has strengths and weaknesses. One strength is that the study design and methodology were intended to minimize potential selection and information biases. Selection bias was minimized by matching cases and controls and by the diagnostic techniques used for cases. The age, sex, risk medical conditions, hospital and the date of admission or outpatient visit were used for matching. Social class was not used for matching, but no differences were observed between cases and controls.

Diagnosis using cultures and PCR increases the capacity to detect IPD [12,38]. If PCR had not been used, two-thirds of cases would not have been detected, and the cases included would not have been representative of hospitalized cases of IPD and could have skewed the study results.

The possibility of information bias was minimized collecting information on vaccination status from personal health records (vaccination card, health card, medical history or the primary healthcare center register). Although investigators collecting this information were not blinded to the status of each case or control, vaccination histories were always collected according to this recorded information.

Likewise, adjustment of age and other variables that differed between cases and controls by conditional logistic regression helped minimize potential confounding.

The sample size is another strength. Only the study by Whitney et al. [20] included a greater number of cases and controls.

The main weakness of our study is the low proportion of cases (8.9%) caused by vaccine serotypes, which made it difficult to obtain significant results in the analysis by specific subgroups. However, this variable could not be controlled.

Our results suggest that the recently licensed conjugate vaccines will increase coverage of IPD-causing serotypes in Catalonia from the 8.9% covered by the PCV7 to 40.3% for the 10-valent vaccine (incorporating serotypes 1, 5 and 7F) and 70.6% for the 13-valent vaccine (additionally incorporating serotypes 3, 6A, and 19A).

In summary, our results confirm those of other observational studies showing high levels of effectiveness of complete vaccination with PCV7 in the prevention of IPD caused by vaccine serotypes in children aged 7–59 months in a population with intermediate vaccination coverages. The small number of cases due to vaccine serotypes in children aged 24–59 months substantially reduced the power of the study in this age group, and explains why the protection observed was not statistically significant.

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Author's contributions: All the authors participated in the design, implementation, analysis and interpretation of the study. A.

Dominguez was the principal investigator, drafted the report and secured funding. L. Salleras, P. Ciruela, S. Hernandez, N. Cardeñosa, J. Batalla and N. Soldevila oversaw data management and conducted the statistical analysis. J.J. García, F. Moraga, M.F. de Sevilla, I. Jordan and F. Coll were the investigators in the clinical services of the hospitals involved. C. Muñoz-Almagro, L. Selva, C. Esteva, G. Codina and A.M. Planes were the microbiologists who performed the laboratory analysis.

Conflict of interest statement: We declare that we have no conflict of interest apart from the following:

P. Ciruela, M.F. de Sevilla, S. Hernandez and J.J. García have received a travel grant from Pfizer. J.J. García has received honoraria for speaking at cientific meetings from Pfizer. F. Moraga has received honoraria for consultancy and speaking at scientific meetings from Pfizer and GSK. L. Salleras has received travel grants for speaking at scientific meetings organized by Sanofi Pasteur MSD, GSK, Novartis vaccines, Pfizer, Crucell and Esteve.

References

[1] Fedson DS, Musher DM. Pneumococcal vaccine. In: Plotkin SA, Orenstein WA, editors. Vaccines. 3rd ed. Philadelphia: WB Saunders; 1999. p. 553–607.
[2] Salleras L, Domínguez A, Navarro JÁ. Vacuna antineumocócica conjugada. In: Salleras L, editor. Vacunaciones preventivas. 2nd ed. Barcelona: Masson; 2003. p. 399–420.
[3] Poland G. The burden of pneumococcal disease: the role of conjugate vaccines. Vaccine 1999;17:1674–9.
[4] Butler JC, Hofmann J, Cetron MS, Elliott JA, Facklam RR, Breiman RF. The continued emergence of drug-resistant *Streptococcus pneumoniae* in the US: an update from the Centers for Disease Control and Prevention's Pneumococcal Sentinel Surveillance System. J Infect Dis 1996;174:986–93.
[5] Black S, Shinefield H, Fireman B, Lewis E, Ray P. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr Infect Dis J 2000;19:187–95.
[6] CDC Advisory Committee on Immunization Practices. Preventing pneumococcal disease among infants and children. Recommendations of the Advisory Committee on Immunization Practices. MMWR Recomm Rep 2000;49(RR-9):1–35.
[7] Butler JC, Breiman RF, Lipman HB, Hofmann J, Facklam RR. Serotype distribution of streptococcus pneumoniae infections among preschool children in the United States, 1978–1994: implications for development of a conjugate vaccine. J Infect Dis 1995;171:885–9.
[8] Salleras L, Fenoll A, Domínguez A, Navas E, Casal J. Cobertura de serotipos de la vacuna antineumocócica conjugada heptavalente, Cataluña, 1997–2002. Vacunas 2003;4:73–9.
[9] Bernaola E, Giménez F, Baca M, De Juan F, Diez J, Garcés M, et al. Vaccination schedule of the Spanish association of pediatrics: recommendations. An Pediatr (Barc) 2009;70:72–82.
[10] Borras E. Study of vaccination coverage in children under 3 years of age in Catalonia (Spain). Doctoral Thesis. University of Barcelona, Spain. Updated 2006, http://www.tdx.cat/handle/10803/2845 [accessed 2.05.2011].
[11] Calbo E, Díaz A, Cañadell E, Fábrega J, Uriz S, Xercavins M, et al. Invasive pneumococcal disease among children in a health district of Barcelona: early impact of pneumococcal conjugate vaccine. Clin Microbiol Infect 2006;12:867–72.
[12] Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 2008;46:174–82.
[13] Salleras L, Domínguez A, Ciruela P, Izquierdo C, Borras E. Impacto de la vacuna neumocócica conjugada heptavalente en una población con valores bajos-intermedios de vacunación. Enf Infecc Microbiol Clin 2009;27:275–7.
[14] Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, Craig AS, et al. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J Infect Dis 2007;9:1346–54.
[15] Ciruela P, Hernández S, Izquierdo C. Epidemiology of invasive pneumococcal disease (2001–2008). Butlletí Epidemiològic de Catalunya 2010;XXX(2):31–7.
[16] Salleras L, Domínguez A, Ciruela P, Izquierdo C, Navas E, Torner N, et al. Changes in serotypes causing invasive pneumococcal disease (2005–2007 vs 1997–1999) in children under 2 years of age in a population with intermediate coverage of the 7-valent pneumococcal conjugated vaccine. Clin Microbiol Infect 2009;15:997–1001.
[17] Salleras L. The emergence of non-vaccine serotypes in invasive pneumococcal disease: a probably multifactorial origin. Vacunas 2008;9(Suppl. 1):3–11.
[18] Obando I, Muñoz-Almagro C, Arroyo LA, Tarrago D, Sanchez-Tatay D, Moreno-Perez D, et al. Pediatric parapneumonic empyema. Spain Emerg Infect Dis 2008;14:1390–7.
[19] Picazo J, Ruiz-Contreras J, Casado-Flores J, Giangaspro E, Del Castillo F, Hernández-Sampelayo T, et al. Relationship between serotypes, age, and clinical presentation of invasive pneumococcal disease in Madrid, Spain, after introduction of the 7-valent pneumococcal conjugate vaccine into the vaccination calendar. Clin Vaccine Immunol 2011;18:89–94.
[20] Whitney CG, Pilishvili T, Farley MM, Schaffner W, Craig AS, Lynfield R, et al. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet 2006;368:1495–502.
[21] Deceuninck G, De Wals P, Bouliannne N, De Serres G. Effectiveness of pneumococcal conjugate vaccine using a 2+1 infant schedule in Quebec, Canada. Pediatr Infect Dis J 2010;29:546–9.
[22] Barricarte A, Castilla J, Gil-Setas A, Torroba L, Navarro-Alonso JA, Irisarri F, et al. Effectiveness of the 7-valent pneumococcal conjugate vaccine: a population-based case-control study. Clin Infect Dis 2007;44:1436–41.
[23] Mahon BE, Hsu K, Karumuri S, Kaplan SL, Mason Jr EO, Pelton SI, et al. Effectiveness of abbreviated and delayed 7-valent pneumococcal conjugate vaccine dosing regimens. Vaccine 2006;24:2514–20.
[24] Ruckinger J, van der Linden M, Reinert RR, von Kries R. Efficacy of 7-valent pneumococcal conjugate vaccination in Germany: an analysis using the indirect cohort method. Vaccine 2010;28:5012–6.
[25] Muñoz-Almagro C, Gala S, Selva L, Jordan I, Tarragó D, Pallares R. DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema. Eur J Clin Microbiol Infect Dis 2011;30:327–35.
[26] Tarragó D, Fenoll A, Sanchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin Microbiol Infect 2008;14:828–34.
[27] American Academy of Pediatrics. 2009 Report of the committee on infectious diseases. 28th ed. Elk Grove Village: American Academy of Pediatrics; 2009.
[28] Office of Population Censuses Surveys. Classification of occupations. London: HMSO; 1980.
[29] Schlesselman JJ. Case-control studies: design, conduct analysis. New York: Oxford University Press; 1982. pp. 144–70.
[30] Hausdorff WP. Invasive pneumococcal disease in children: geographic and temporal variations in incidence and serotype distribution. Eur J Pediatr 2002;161(Suppl. 2):135–9.
[31] Grijalva CG, Nuorti JP, Zhu Y, Griffin MR. Increasing incidence of empyema complicating childhood community-acquired pneumonia in the United States. Clin Infect Dis 2010;50:805–13.
[32] Koshy E, Murray J, Bottle A, Sharland M, Saxena S. Impact of the seven-valent pneumococcal conjugate vaccination (PCV7) programme on childhood hospital admissions for bacterial pneumonia and empyema in England: national time-trends study, 1997–2008. Thorax 2010;65:770–4.
[33] Pelton SI, Weycker D, Klein JO, Srutton D, Ciuryla V, Oster G. 7-valent pneumococcal conjugate vaccine and lower respiratory tract infections: effectiveness of a 2-dose versus 3-dose primary series. Vaccine 2010;28:1575–82.
[34] Esteva C, Selva L, de Sevilla MF, García-García JJ, Pallarés R, Muñoz-Almagro C. *Streptococcus pneumoniae* serotype 1 causing invasive disease among children in Barcelona over a 20-year period (1989–2008). Clin Microbiol Infect 2011;17:1441–4.
[35] Spijkerman J, van Gils EJ, Veenhoven RH, Hak E, Yzerman EP, van der Ende A, et al. Carriage of *Streptococcus pneumoniae* 3 years after start of vaccination program, the Netherlands. Emerg Infect Dis 2011;17:584–91.
[36] Gonçalves G. Herd immunity: recent uses in vaccine assessment. Expert Rev Vaccines 2008;7:1493–506.
[37] Centers for Disease Control Prevention. Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease. United States, 1998–2003. MMWR Morb Mortal Wkly Rep 2005;54:893–7.
[38] Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, de Martino M, et al. Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. Med Microbiol 2008;57:1205–12.

lates, based on the meropenem susceptibility test result and the use of meropenem disks supplemented with APB, cloxacillin or DPA. These tests will enable routine laboratories to identify, with high confidence levels, those *P. aeruginosa* isolates suspected of producing either KPC or MBL carbapenemases.

Transparency Declaration

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References

1. Miriagou V, Cornaglia G, Edelstein M *et al.* Acquired carbapenemases in gram-negative bacterial pathogens: detection and surveillance issues. *Clin Microbiol Infect* 2010; 16: 112–122.
2. Carmeli Y, Akova M, Cornaglia G *et al.* Controlling the spread of carbapenemase-producing gram-negatives: therapeutic approach and infection control. *Clin Microbiol Infect* 2010; 16: 102–111.
3. Giske CG, Gezelius L, Samuelsen O *et al.* A sensitive and specific phenotypic assay for detection of metallo-beta-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. *Clin Microbiol Infect* 2011; 17: 552–556.
4. Clinical Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: 21st informational supplement*. CLSI document M100-S21. Wayne, PA: CLSI, 2011.
5. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
6. Pasteran F, Mendez T, Guerriero L *et al.* Sensitive screening tests for suspected class A carbapenemase production in species of Enterobacteriaceae. *J Clin Microbiol* 2009; 47: 1631–1639.
7. Pasteran F, Mendez T, Rapoport M *et al.* Controlling false-positive results obtained with the Hodge and Masuda assays for detection of class a carbapenemase in species of enterobacteriaceae by incorporating boronic acid. *J Clin Microbiol* 2010; 48: 1323–1332.
8. Juan C, Maciá MD, Gutiérrez O *et al.* Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob Agents Chemother* 2005; 49: 4733–4738.
9. Papanicolaou GA, Medeiros A, Jacoby G. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 1990; 34: 2200–2209.
10. Jiang X, Zhang Z, Li M *et al.* Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2006; 50: 2990–2995.
11. Samuelsen O, Toleman MA, Sundsfjord A *et al.* Molecular epidemiology of metallo-beta-lactamase-producing Pseudomonas aeruginosa isolates from Norway and Sweden shows import of international clones and local clonal expansion. *Antimicrob Agents Chemother* 2010; 54: 346–352.

Streptococcus pneumoniae serotype 1 causing invasive disease among children in Barcelona over a 20-year period (1989–2008)

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Abstract

Fifty-six isolates of serotype 1 were identified during a 20-year prospective study (1989–2008), including all children with culture-proven invasive pneumococcal disease (IPD) admitted to a children's hospital in Barcelona. Forty-eight of them (85.7%) were in children aged >2 years. Complicated pneumonia (n = 28) and non-complicated pneumonia (n = 20) were the main clinical presentations. The frequency of serotype 1 IPD increased from 1999–2003 to 2004–2008: 1.2 to 4.4 episodes/100 000 children (p <0.001). The ST306 clone were identified in 70.4% of isolates. As IPD caused by serotype 1 is mainly detected in older children, a vaccination programme for children >2 years should be considered.

Keywords: Invasive pneumococcal disease, MLST, resistance, serotype 1, *Streptococcus pneumoniae*

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Invasive pneumococcal disease (IPD) is associated with high rates of morbidity and mortality in children. Serotype 1 is one of the most prevalent invasive serotypes of *Streptococcus pneumoniae*. In recent years, several studies have found an increase in IPD caused by serotype 1 in different countries [1–5].

In this study, we analysed changes in the epidemiology of serotype 1 among children in Barcelona, and determined the clonal composition of invasive strains of serotype 1.

We carried out a 20-year prospective study that included all children and adolescents aged <18 years with IPD who were admitted to Sant Joan de Déu Hospital.

This is a 345-bed children's teaching hospital located in Barcelona that was responsible for 18.5% of all paediatric hospitalizations in Catalonia, Spain in1999–2003 and 18.3% in 2004–2008. A detailed description of the methodology used in this study has been reported elsewhere [6].

IPD was defined as the presence of clinical findings of infection together with isolation of *S. pneumoniae* in a blood sample, cerebrospinal fluid sample, or any other sterile fluid sample. Pneumococcal strains were serotyped by the Quellung reaction, and clonal analysis was performed by multilocus sequence typing (MLST).

For statistical analysis, we used the chi-square test or Fisher's exact test, when appropriate, to compare proportions, and Student's *t*-test to compare means. Rates of IPD (episodes/100 000 population) were calculated from the annual estimates of the paediatric population obtained from the Department of Statistics in Catalonia [7].

A total of 347 episodes of IPD were identified. Fifty-six of 344 episodes (16.3%) with the serotype available were caused by serotype 1. According to age group, eight episodes (14.3%) caused by serotype 1 were in children aged <2 years, 19 episodes (33.9%) were in children aged 2–4 years, and 29 episodes (51.8%) were in children and adolescents aged 5–17 years. Table 1 shows the characteristics of patients with IPD caused by serotype 1 vs. other serotypes.

On comparison of the proportion of serotype 1 with respect to the total episodes of IPD, there was a statistically significant increase in serotype 1 throughout the study period: one of 51 episodes (2%) in 1989–1993; five of 58 episodes (8.6%) in 1994–1998; 11 of 74 episodes (14.9%) in 1999–2003; and 39 of 164 episodes (23.8%) in 2004–2008 (chi-square test for trend, p <0.001).

Fig. 1 shows the increase in incidence of IPD among children throughout the study by age group. In addition, a statistically significant increase was observed on comparison of the rates of serotype 1 per 1000 hospital admissions between 1999–2003 and 2004–2008 (13 vs. 45.1 per 1000 admissions; Fisher's-exact test, p <0.001) and comparison of the rates of serotype 1 per 1000 blood cultures between the two periods (12.8 vs. 79.2 per 1000 blood cultures; Fisher's-exact test, p <0.001).

TABLE 1. Clinical manifestations of invasive pneumoccocal disease (IPD) in children

	Episodes (%), serotype 1 (*n* = 56)	Episodes (%), other serotypes (*n* = 288)	p-value
Age (months), mean (±SD)	62.3 (42.0)	34.5 (39.2)	p <0.001[d]
Sex			
Female	27 (48.2)	115 (39.9)	NS; p 0.44[e]
Male	29 (51.8)	173 (60.1)	
PCV7 vaccination[a]	5 (9.4)	25 (9.4)	NS; p 0.78[e]
Clinical manifestations of IPD[b]			
Meningitis	1 (1.8)	70 (24.5)	p <0.001[e]
Pneumonia (overall)	48 (85.7)	77 (26.9)	
With empyema	28	40	
Without empyema	20	37	
Bacteraemia/sepsis	5 (8.9)	100 (34.9)	
Appendicitis	2 (3.6)	9 (3.1)	
Arthritis	0	28 (9.8)	
Others	0	2 (0.7)	
PICU admission[c]	3 (5.3)	72 (27.6)	p <0.001[f]
Mortality	0	13 (4.5)	NS; p 0.1[f]

NS, not significant; PCV7, heptavalent pneumococcal conjugate vaccine; PICU, paediatric intensive care unit; SD, standard deviation.
[a]PCV7 vaccination status with was available in 319 patients (53 with serotype 1 and 266 with other serotypes).
[b]Clinical manifestations of IPD were known in 342 patients (56 with serotype 1 and 286 with other serotypes).
[c]PICU admission status was available in 316 patients (56 with serotype 1 and 260 with other serotypes).
For statistical analysis, we used the chi-square test or Fisher's exact test, when appropriate, to compare proportions, and Student's *t*-test to compare means: [d]Student's *t*-test, [e]chi-square test, [f]Fisher's exact test.

According to the meningeal breakpoints of the CLSI [8], all serotype 1 isolates were susceptible to penicillin and cefotaxime during the study period, but three strains (7.7%) detected in 2004–2008 were resistant to erythromycin, and two (5.1%) were also resistant to tetracycline and chloramphenicol.

Molecular analysis by MLST was performed in 55 of 56 (98.2%) serotype 1 isolates. Overall, there were five different sequence types (STs) expressing serotype 1, including three PMEN clones: ST306 (Sweden ^{1}-28) in 39 isolates (71%), ST304 (Sweden ^{1}-40) in 11 isolates (20%), and ST217 (Sweden ^{1}-27) in one isolate (1.8%). In addition, ST228, which is a double-locus variant of Sweden ^{1}-28, was detected in three isolates (5.5%), and a new MLST profile, ST2376, which is a single-locus variant of Sweden ^{1}-28, was detected in one isolate. Serotype 1 was detected for the first time in 1994–1998, and the first STs detected were ST304 and ST228. All ST306 isolates were detected after January 2000.

Some studies have suggested that certain surface or subsurface proteins of pneumococci may contribute significantly to the pathogenesis and virulence of some strains [9]. One recently identified pneumococcal virulence determinant is the pneumococcal serine-rich repeat protein (PsrP). Animal models

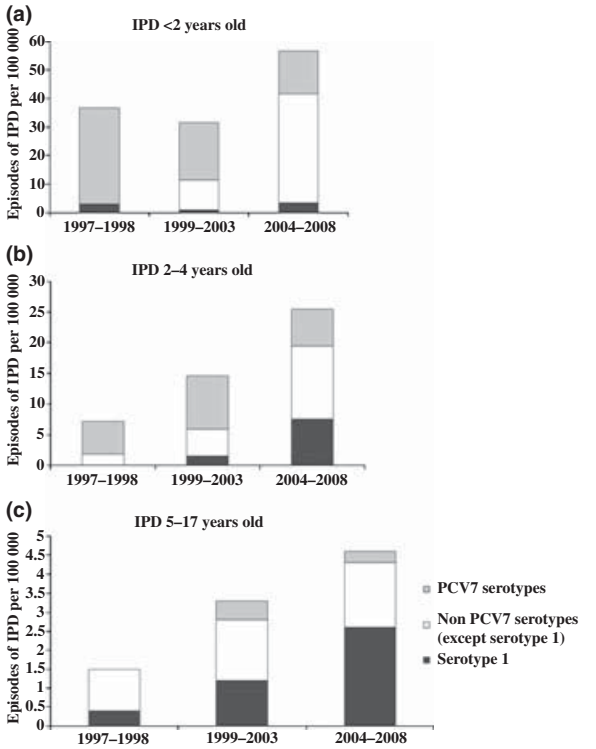

FIG. 1. Incidence of invasive pneumococcal disease (IPD) among children throughout the study period by age group. On comparison of the pre-vaccine period, the early heptavalent pneumococcal conjugate vaccine (PCV7) period (1999–2003) and the late PCV7 period (2004–2008), there was no statistically significant increase in serotype 1 in children aged <2 years (1 vs. 3.5 per 100 000 population; Fisher's exact test, p 0.1), a statistically significant increase in serotype 1 was observed in children aged 2–4 years (1.5 vs. 7.5 per 100 000 population; Fisher's exact test, p 0.010), and there was a trend for an increase in children and adolescents aged 5–17 years (1.2 vs. 2.6 per 100 000 population; Fisher's exact test, p 0.050).

have shown that PsrP mediates attachment to lung cells [10,11]. In addition, we recently reported that PsrP is highly prevalent in children with pneumonia and is strongly associated with clonal type. In that study, PsrP was detected in all ST306 isolates studied (n = 88) [12]. Therefore, the introduction of ST306 in our geographical area was associated with the introduction of this virulence factor that could be related, in part, to an increased ability of pneumococci to cause pneumonia.

Overall, younger children are a higher risk for IPD, owing to the high rate of pneumococcal nasopharyngeal colonization. However, serotype 1 is rarely found in the nasopharynx, and it is a serotype with a low rate of colonization [13]. Nevertheless, in our series, the incidence of serotype 1 was significantly higher in older children than in younger children (<2 years). This observation has also been reported by other authors [14]. The low prevalence of serotype 1 in carriers could also be an explanation for the low rate of antibiotic resistance and the relative stability of clonal composition. We have detected five different STs, which confirms the high homogeneity of this serotype described by other authors [13] in comparison with the high diversity of other serotypes, such as serotype 19A [15].

The presence of some clones in a specific geographical area depends on several factors, such as the characteristics of the people and social factors in that community and/or the virulence of the clonal types [16]. Other authors have reported that ST306 is a predominant clone in continental Europe [5,17,18], whereas other STs, such as ST217, are more common in North America, England, Canada, and Gambia [14,19].

In conclusion, our study shows an increase in IPD caused by serotype 1 among children in Barcelona. Implementation of the new conjugate vaccines that include serotype 1 is urgently needed to reduce the burden of IPD. As IPD caused by serotype 1 is mainly detected in older children, vaccination programmes for children aged >2 years should also be considered.

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Transparency Declaration

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References

1. Henriques Normak B, Kalin M, Ortqvist A *et al.* Dynamics of penicillin-susceptible clones in invasive pneumococcal disease. *J Infect Dis* 2001; 184: 861–869.
2. Konradsen HB, Kaltoft MS. Invasive pneumococcal infections in Denmark from 1995 to 1999: epidemiology, serotypes, and resistance. *Clin Diagn Lab Immunol* 2002; 9: 358–365.

3. McChlery SM, Scott KJ, Clarke SC. Clonal analysis of invasive pneumococcal isolates in Scotland and coverage of serotypes by licensed conjugate polysaccharide pneumococcal vaccine: possible implications for UK vaccine policy. *Eur J Clin Microbiol Infect Dis* 2005; 24: 262–267.
4. Byington C, Korgenski K, Daly J, Ampofo K, Pavia A, Mason EO. Impact of pneumococcal conjugate vaccine on pneumococcal parapneumonic empyema. *Pediatr Infect Dis J* 2006; 25: 250–254.
5. Obando I, Muñoz-Almagro C, Arroyo LA *et al.* Pediatric parapneumonic empyema, Spain. *Emerg Infect Dis* 2008; 14: 1390–1397.
6. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. *Clin Infect Dis* 2008; 46: 174–182.
7. Web de l'Estadística Oficial de Catalunya. Institut d'Estadística de Catalunya. Available at: http://www.idescat.net (accessed 25 May 2009)..
8. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial susceptibility testing*. Eighteenth informational supplement. CLS document M100-S18. Wayne, PA: CLSI, 2008.
9. Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. *Nat Rev Microbiol* 2008; 6: 288–301.
10. Obert C, Sublett J, Kaushal D *et al.* Identification of a candidate *Streptococcus pneumoniae* core genome and regions of diversity correlated with invasive pneumococcal disease. *Infect Immun* 2006; 74: 4766–4777.
11. Shivshankar P, Sanchez C, Rose LF, Orihuela CJ. The *Streptococcus pneumoniae* adhesin PsrP binds to keratin 10 on lung cells. *Mol Microbiol* 2009; 73: 663–679.
12. Muñoz-Almagro C, Selva L, Sanchez CJ *et al.* PsrP, a protective pneumococcal antigen, is highly prevalent in children with pneumonia and is strongly associated with clonal type. *Clin vaccine Immunol* 2010; 17: 1672–1678.
13. Bruggemann AB, Spratt BG. Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates. *J Clin Microbiol* 2003; 41: 4966–4970.
14. Gonzalez B, Hilten K, Kaplan S, Mason EO Jr, the US Pediatric Multicenter Pneumococcal Surveillance Study Group. Clonality of *Streptococus pneumoniae* serotype 1 isolates from paediatric patients in the United States. *J Clin Microbiol* 2004; 42: 2810–2812.
15. Muñoz-Almagro C, Esteva C, de Sevilla MF, Selva L, Gene A, Pallares R. Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. *J Infect* 2009; 59: 75–82.
16. Chiou A, Sgambatti S, Almeida SC, Cobo R, Andrade AL, De Cunto MC. Molecular assessment of invasive *Streptococcus pneumoniae* serotype 1 in Brazil: evidence of clonal replacement. *J Med Microbiol* 2008; 57: 839–844.
17. Marimon JM, Ercibengoa M, Alonso M, Zubizarreta M, Pérez-Trallero E. Clonal structure and 21-year evolution of *Streptococcus pneumoniae* serotype 1 isolates in northern Spain. *Clin Microbiol Infect* 2009; 15: 875–877.
18. Nunes S, Sá-Leáo R, Pereira LC, Lencastre H. Emergence of a serotype 1 *Streptococcus pneumoniae* lineage colonising healthy children in Portugal in the seven-valent conjugate vaccination era. *Clin Microbiol Infect* 2008; 14: 82–84.
19. Martín A, Ishrat H, Timothy A *et al.* Seasonality and outbreak of a predominant *Streptococcus pneumoniae* serotype 1 clone from the Gambia: expansion of ST217 hypervirulent clonal complex in West Africa. *BMC Microbiol* 2008; 8: 198.

Outbreak of KPC-3-producing, and colistin-resistant, *Klebsiella pneumoniae* infections in two Sicilian hospitals

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Abstract

We report the first outbreak caused by colistin-resistant *Klebsiella pneumoniae* producing KPC-3 carbapenamase in two Italian hospitals. This spread occurred in 1 month, and was caused by eight colistin-resistant and carbapenem-resistant *Klebsiella pneumoniae* isolates from eight patients. A further three isolates were obtained from the intestinal tract and pharyngeal colonization. All isolates were multidrug-resistant (MDR), including being resistant to colistin, but they were susceptible to gentamicin and tigecycline. PCR detection showed that all isolates harboured the bla_{KPC-3} gene associated with bla_{SHV-11}, bla_{TEM-1} and bla_{OXA-9}. All *K. pneumoniae* isolates, genotyped by pulsed-field gel electrophoresis and multilocus sequence typing, belonged to the same sequence type (ST)258 clone. From our data and a review of the international literature, *K. pneumoniae* ST258 seems to be the most widespread genetic background for KPC dissemination in Europe.

Keywords: Colistin resistance, colonization, *Klebsiella pneumoniae*, KPC-3, ST258

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Serotypes and clones causing invasive pneumococcal disease before the use of new conjugate vaccines in Catalonia, Spain

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Summary *Objectives:* The objective of this study was to learn the serotype distribution and clonal composition of pneumococci causing invasive pneumococcal disease (IPD) in children and adults in Spain before the introduction of new 10-valent (PCV10) and 13-valent (PCV13) conjugate vaccines.
Methods: This is a 1-year prospective study including all patients with culture-proved IPD admitted to 30 medical centers in Catalonia, Spain, during the year 2009.
Results: A total of 614 episodes of IPD occurred in 612 patients. The rates of IPD were highest in children aged <24 months and adults >64 years (64.5 and 44.7 per 100,000 population). The burden of disease was mainly due to pneumonia in all age ranges. 609 of 614 strains were serotyped and 47 different serotypes were found. Among the 609 IPD cases with known serotype, 12.2% were caused by PCV7 serotypes, 51% by PCV10 serotypes, and 71.7% by PCV13 serotypes. 608 of 614 isolates were characterized by MLST. The main clonal types detected were ST306, CC191 and CC230.

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Conclusions: PCV13 conjugate vaccine offers good coverage against IPD in Catalonia, Spain. However, the high genetic diversity of pneumococci highlights the importance of molecular surveillance systems for monitoring IPD during the vaccination period.
Summary: This study shows that 13-valent conjugate vaccine offers good coverage against invasive pneumococcal disease in children and adults in Spain. However, the high genetic diversity of pneumococci highlights the importance of molecular surveillance systems for monitoring IPD during the vaccination period.

Introduction

Invasive pneumococcal disease (IPD) is an important health problem. *Streptococcus pneumonie* annually kills more than 800,000 children under 5 years of age.[1] This pathogen is directly responsible for as many child deaths as AIDS, malaria, and tuberculosis combined.[2] Many of these deaths could be prevented with vaccination. In the year 2000, a protein-polysaccharide conjugate vaccine against the serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (PCV7) was licensed for use in infants and young children in the United States. PCV7 was introduced in Spain in 2001. However, this vaccine is not currently being subsidized by the Spanish Health Service, except for the regional area of the Community of Madrid which included this vaccine in the childhood systematic immunization schedule in October 2006, replacing it with the 13-valent conjugate vaccine in June 2010.

PCV7 has been proven to be safe, immunogenic and effective in preventing pneumococcal disease caused by vaccine serotypes in children.[3,4] In addition, PCV7 also prevents IPD in adults and non-vaccinated children through an indirect effect (herd immunity) on pneumococcal transmission.[5] Before the introduction of PCV7, serotypes included in PCV7 covered 65—80% of serotypes associated with invasive pneumococcal disease among young children in Western industrialized countries.[6] However, this coverage is much lower in many developing countries, where more than 90 percent of deaths attributable to pneumococcal diseases occur.[1,6,7] At present, despite the effectiveness of PCV7, which has allowed a significant decrease in invasive pneumococcal disease[8—10], the emergence of complicated pneumococcal pneumonia by non-PCV7 serotypes, especially in children, is a fact in many geographical areas.[11—16] These data imply that pneumococcal disease prevention must be accelerated and a new generation of vaccines is urgently needed.

The serotype coverage of 10-valent conjugate vaccine (PCV10) which included the seven serotypes of PCV7 plus serotypes 1, 5 and 7F, and especially PCV13 (PCV10 plus additional serotypes 3.6A and 19A), is expected to provide greater protection against pneumococcal infection, due to the greater number of serotypes covered by these vaccines. In addition, new conjugate vaccines offer coverage against serotypes with high invasive disease potential such as 1, 5 and 7F.[17] PCV10 was authorized in our country in April 2009 and PCV13 in January 2010.

Geographical and temporary differences of main clonal types of pneumococci and their associated serotypes have been widely reported.[6] An important increase in ST306 expressing serotype 1 has recently been detected[14,15,18] in Spain and other European countries, while this clonal type and serotype is minor in the U.S.[19] Knowledge of prevalent clonal types is interesting to improve understanding of the ability of pneumococci to spread in a population.

The objective of this study was to learn the distribution of serotypes and clonal composition of *S. pneumoniae* isolates causing IPD in children and adults in Spain before the introduction of the new 10-valent and 13-valent conjugate vaccines.

Patients and methods

Study setting and design

This is a prospective study including all patients with invasive pneumococcal disease (IPD) attended in 30 health centers in Catalonia, Spain, from January 1 to December 31, 2009. In Catalonia, with a population of around 7 million and 1.2 million persons aged 18 years or younger, these 30 health centers captured 40.5% (395,361 of 977, 211) of all hospital admissions and, specifically, 63% (61,575 of 98,216) of paediatric hospital admissions during 2009.[20]

Case definition

Invasive pneumococcal disease (IPD) was defined as the presence of clinical findings of infection together with isolation of *S. pneumoniae* by culture in any normal sterile fluid. IPD was classified according to International Classification of Disease, Ninth Revision (ICD-9), specific for diseases caused by *S. pneumoniae* including occult bacteremia/sepsis, meningitis, pneumonia, parapneumonic empyema, peritonitis and arthritis.

Microbiological identification, serotyping and antimicrobial susceptibility

Isolates were identified in the different centers by standard microbiological methods that included Gram stain morphology, optochin sensitivity test and bile solubility test. After this presumptive identification, strains were delivered to the Molecular Microbiology Department of the University Hospital Sant Joan de Deu, which is the center designated by the government of Catalonia, Spain for molecular surveillance of invasive pneumococcal disease. Upon arrival in this laboratory, isolates were serotyped by a published multiplex-PCR assay that allows rapid detection of 24 serotypes (1, 3, 4, 5, 6A, 6B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/BC, 23A, and 23F)[21].

All strains were also sent to the National Pneumococcus Reference Center of Majadahonda, Madrid, Spain, to complete serotype study by Quellung reaction and to determine

the MICs of penicillin and other antibiotics by Agar dilution technique. Antibiotic susceptibilities were defined according to the 2008 meningeal breakpoints suggested by the Clinical Laboratory Standards Institute.[22] We used meningeal breakpoints for non-meningitis infections for the purpose of epidemiological surveillance because isolates with diminished susceptibility to penicillin are increasing in certain serotypes such as 19A[23,24] and non-meningeal breakpoints may underestimate this phenomenon. Isolates with intermediate or high-level resistance were defined as non-susceptible. Non-susceptibility to $\geq$ 3 antibiotic classes was considered multidrug resistance.

Clonal analysis

MLST was performed as reported elsewhere.[25] The assignment of alleles and sequence types (ST) was carried out using the software at the pneumococcal web page www.mlst.net. Analysis of ST and assignment to clonal complex were performed with the eBURST program.[26] STs that shared six of seven allelic (single locus [SLV]) variants were considered a clonal complex. Genetic diversity of clonal population was estimated using Simpson's numerical index of discrimination (SID) www.comparingpartitions.info

Statistical analysis

We used the X^2 test or Fisher's exact test to compare proportions. Non-parametric Kruskal–Wallis test was used to compare differences in susceptibility of strains to different antimicrobial agents for each age group. Statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS), and Epi Info, version 6.0 (Centers for Disease Control and Prevention). We calculated 95% CIs, and 2-sided *P* values <.05 were considered to be statistically significant.

Results

A total of 614 episodes of invasive pneumococcal disease (IPD) occurred in 612 patients during the study period. One child with CSF fistulae had 2 episodes of meningitis with serotypes 19A and 6A and another child had 2 episodes of pneumonia with serotype 24F.

There were 340 males (55.5%) and 272 females (44.5%), with a mean age of 45.3 years (range 22 days–97 years). Seventy-one episodes (11.6%) were in children < 2 years old, 59 episodes (9.6%) were in children 2–4 years old, 46 episodes (7.5%) in children 5–17 years old, 226 episodes (36.8%) in adults 18–64 years old, and 212 episodes (34.5%) in adults > 64 years old. All 614 episodes of IPD had positive cultures for *S.pneumoniae* (one or more): blood 545, pleural fluid 41, CSF 31, joint fluid 7, peritoneal fluid 3 and pulmonary biopsy 1. The clinical manifestation was pneumonia in 481 (78.3%), 62 of them with empyema, occult bacteraemia/sepsis 77 (12.6%), meningitis 40 (6.5%), appendicitis/peritonitis 8 (1.3%), arthritis 7 (1.1%), and pericarditis 1 (.2%).

Vaccination status was registered in 53 of 71 children less than 2 years of age and in 45 of 59 children 2–4 years old; 60.3% and 77.7%, respectively, had been vaccinated with PCV7. IPD was caused by non-vaccine serotypes in all vaccinated children.

Incidence of IPD according to age group and clinical manifestation

Overall rates were highest in children aged <2 years, children 24–59 months and adults >64 years (64.5, 28.8 and 44.7 per 100,000 population, respectively); among children aged 5–17 years and adults 18–64 years, the rates decreased (8.9 and 12.3 per 100,000 population, respectively) (Table 1). The burden of disease was mainly due to pneumonia in all age ranges. Complicated pneumonia with empyema was statistically significantly higher in children <18 years than in adult patients: 5.2 vs .8 per 100,000 population (an increase of 538 (IC 95% 272–995) $P < 0.001$).

Serotype distribution of invasive isolates

609 of 614 strains were serotyped (99.2%) and 47 different serotypes were found. Among the 609 IPD cases with known serotype, 75 (12.2%) were caused by serotypes contained in PCV7 vaccine, 313 (51%) contained in PCV10 vaccine, and 437 (71.7%) contained in PCV13 vaccine. The coverage of these three vaccines was 11.9%, 51.9% and 71.8% among isolates detected only in blood, 10.3%, 59% and 84.6% among isolates from pleural fluid and 16.7%, 36.7% and 60% among isolates from CSF, respectively. Table 2 shows the potential coverage of conjugate vaccines according to clinical diagnosis and age group.

Serotypes included in PCV7 were more frequent in adult patients (>17 years) vs children and adolescents (<18 years): 14.3% (62 of 434 episodes in >17 years) vs 7.4% (13 of 175 episodes in <18 years) ($P = 0.02$). In contrast, serotypes included in PCV13 were more frequent in children vs adults: 82.3% (144 of 175 episodes) vs 67.5% (293 of 434 episodes) ($P < 0.001$). The proportions of PCV10 serotypes were also higher in children than adults: 60% (105 episodes) in children vs 47.9% (208 episodes) in adults ($P = 0.009$).

Overall, serotype 1 was the main prevalent type in our series, with 137 episodes (22.5%), followed by serotype 7F, with 76 episodes (12.5%), serotype 19A, with 68 episodes (11.2%), and serotype 3, with 38 episodes (6.2%). Fig. 1 shows the main serotypes detected according to age group.

Antimicrobial susceptibility

Study according to meningeal breakpoints

The rates for all antimicrobial drugs tested (Table 3) were significantly different according to patient age: children younger than 24 months old and adults >64 years of age had the lowest percentage of susceptible strains.

Overall, the percentage of penicillin non-susceptible isolates was 18.7%, (115 of 614 isolates), cefotaxime 9.6% (58 of 614 isolates including 7 fully resistant), erythromycin 18.3% (111 of 606 isolates), choramphenicol 4.3% (26 of 603 isolates), tetracycline 19.6% (118 of 602 isolates), and levofloxacin .8% (5 of 604 isolates).

The serotypes that caused the most penicillin non-susceptible related IPD were serotype 19A (33.9%), type 14 (26.1%), type 24F (8.7%) and type 23B (5.2%). Fig. 2 shows the distribution of serotypes among strains not

Table 1 Rates of invasive pneumococcal disease (IPD) in children and adults according to clinical manifestation and age.

	Number of Episodes	Rate Episodes per 100,000 population*
Overall	614	20.3
Children < 2 years		
All IPD	71	64.5
Meningitis	8	7.3
Pneumonia	37	33.6
Empyema	11	10.0
Occult bacteremia/sepsis	24	21.8
Others	2	1.8
Children 2—4 years old		
All IPD	59	28.8
Meningitis	1	.5
Pneumonia	51	24.9
Empyema	21	10.3
Occult bacteremia/sepsis	7	3.4
Others	0	.0
Children 5—17 years old		
All IPD	46	8.9
Meningitis	8	1.6
Pneumonia	33	6.4
Empyema	11	2.1
Occult bacteremia/sepsis	2	.4
Others	3	.6
Adults 18—64 years old		
All IPD	226	12.3
Meningitis	9	.5
Pneumonia	188	10.3
Empyema	8	.4
Occult bacteremia/sepsis	24	1.3
Others	5	.3
Adults >64 years old		
All IPD	212	44.7
Meningitis	14	2.9
Pneumonia	172	36.2
Empyema	11	2.3
Occult bacteremia/sepsis	20	4.2
Others	6	1.3

*Incidence: episodes per 100,000 population living in the reference geographical area of 30 hospitals according to data from "Catalonian Institute of Statistics" (www.idescat.net).
Others: arthritis, appendicitis and pericarditis.

susceptible to penicillin and other antimicrobial agents. Among PCV7, PCV10 and PCV13 isolates the percentage of resistant strains was 60.8%, 14.8% and 19.8%, respectively.

Study according to non-meningeal breakpoints

Only one of the 614 strains (.2%) was intermediately penicillin resistant (MIC 4 μg/mL), and none (0%) was fully penicillin resistant according to non-meningeal breakpoints.

Clonal distribution of isolates

Among 609 isolates with available serotype, MLST study was performed in 608 isolates (one non-viable strain). Overall, 154 sequence types (ST) were identified, 39 of which were new STs (3 of them, ST5193, ST5194 and ST5195 carried new alleles). Great genetic diversity was found in the entire collection and this was not significantly different between PCV7 isolates and non-PCV7 isolates: Simpson's index of diversity of 91.6% (95% CI, 86.1—97.1) and 93.3% (95%CI, 92.0—94.7) respectively.

eBURST analysis using the stringent 6/7 identical loci definition grouped these 154 STs into 30 clonal complexes and 78 singletons (supplemental material). Seven CCs accounted for 50.3% of the total collection: ST 306 ($n = 106$), CC191 ($n = 72$), CC230 ($n = 30$), CC156 ($n = 27$), ST304 ($n = 25$), CC1223 ($n = 25$) and CC180 ($n = 21$).

Comparative analysis of our serotype and ST results with those published in the MLST database showed that 20 of our STs expressed different previously reported serotypes: ST 1012 (serotype 8), ST97 (serotype 21), ST393 (serotype 25), ST433 (serotype 28),ST2690 (serotype 29), ST198 (serotype 29), ST1684 (serotype 31), ST63 (serotype 38), ST62

Table 2 Proportion of episodes of invasive pneumococcal disease (IPD) caused by serotypes included in PCV7, PCV10 and PCV13 conjugate vaccines according to clinical diagnosis and age group.

	Number of Patients	% IPD by vaccine serotypes		
		PCV7	PCV10	PCV13
Children < 2 years				
All IPD	71	8.5	39.4	73.2
Meningitis	8	12.4	25	50
Pneumonia	37	4	43.2	83.8
Empyema	11	18.2	54.5	90.9
Occult bacteremia/sepsis	24	4.2	41.7	66.7
Others	2	0	0	50
Children 2—4 years old				
All IPD	59	5	72.9	88.1
Meningitis	1	0	100	100
Pneumonia	51	3.9	76.5	92.2
Empyema	21	4.8	66.7	100
Occult bacteremia/sepsis	7	14.3	42.9	57.1
Others	0	0	0	0
Children 5—17 years old				
All IPD	45	8.9	75.6	88.9
Meningitis	8	25	25	62.5
Pneumonia	32	3.1	87.5	96.9
Empyema	11	0	90.9	100
Occult bacteremia/sepsis	2	0	100	100
Others	3	33.3	66.7	66.7
Adults 18—64 years old				
All IPD	223	12.1	59.6	72.2
Meningitis	9	22.2	44.4	66.7
Pneumonia	185	12.4	65.4	76.2
Empyema	7	28.6	85.7	85.7
Occult bacteremia/sepsis	24	8.3	29.2	54.2
Others	5	0	20	20
Adults >64 years old				
All IPD	211	16.6	35.5	62.6
Meningitis	14	28.6	50	57.1
Pneumonia	171	13.5	32.2	62.6
Empyema	11	9.1	27.3	54.5
Occult bacteremia/sepsis	20	30	50	70
Others	6	33.3	50	50

(serotype 11F), ST433 (serotype 15B), ST 1167 (serotype 19A), ST 876 (serotype 19A), ST 109 (serotype 23F), ST 439 (serotype 23B), ST230 (serotype 24B), ST224 (serotype 6C), ST4310 (serotype 6C), ST1483 (serotype 9N), ST67 (serotype 9N), and ST 280 (serotype 9N). Of note, 17 of these 20 STs expressed non-PCV13 vaccine serotypes.

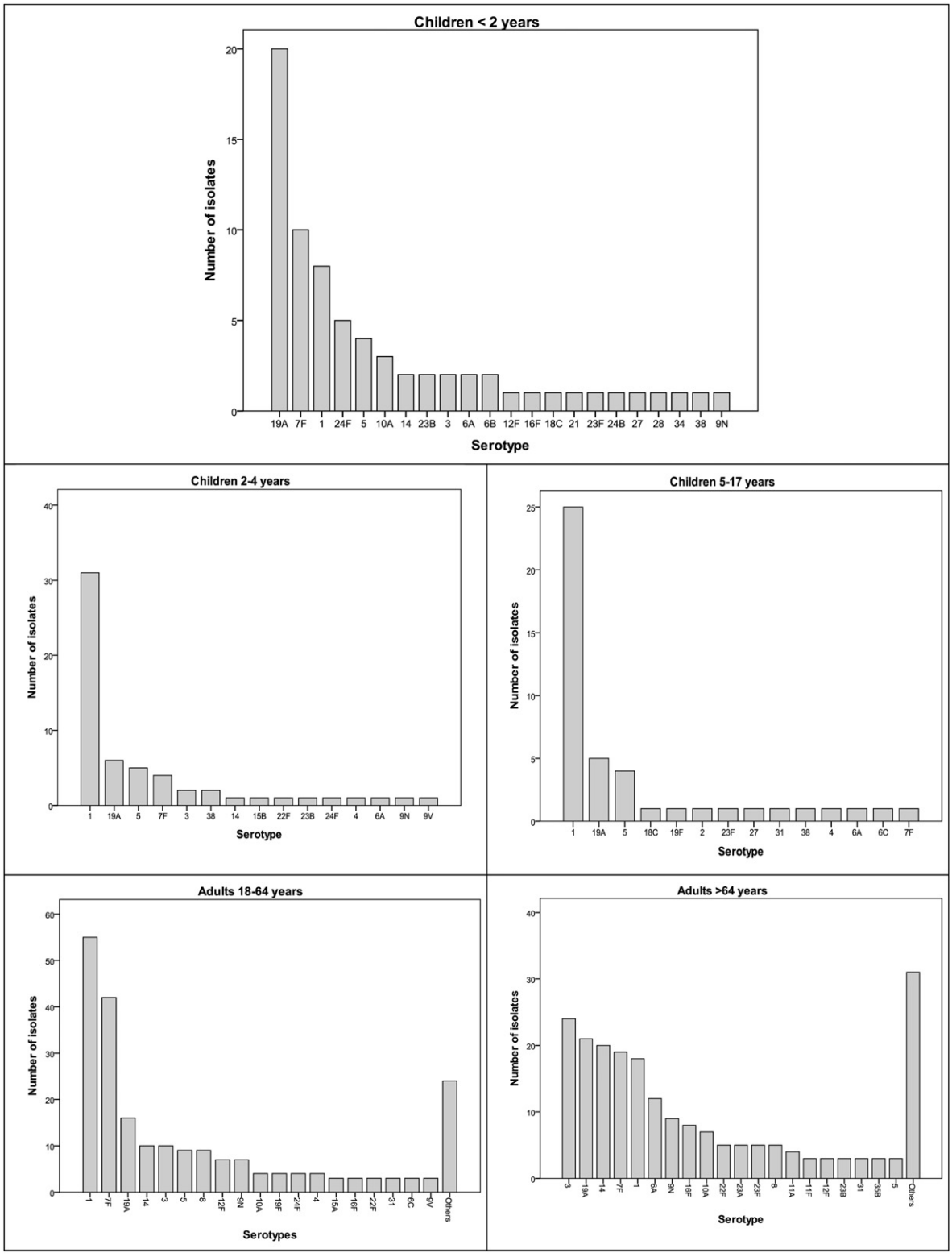
Five clones accounted for 88.6% (31 of 35) of total penicillin non-susceptible strains isolated from children less than 18 years of age: CC230 ($n = 14$), ST320 ($n = 6$), CC2013 ($n = 6$), ST 2372 ($n = 3$) and CC156 ($n = 2$). Twenty of these 31 strains expressed serotype 19A, and the rest serotype 24F (5 strains belonged to CC230), serotype 24B (1 strain belonged to CC230), serotype 23B (3 strains belonged to ST2372), and serotype 14 (2 strains belonged to CC156).

Among penicillin non-susceptible strains isolated from adults, 7 clonal types accounted for 80% (64 of 80 strains): CC156 ($n = 25$), CC230 ($n = 16$), CC63 ($n = 8$), CC2013 ($n = 5$), CC81 ($n = 4$), ST2372 ($n = 3$), and ST320 ($n = 3$). The majority of these 7 clonal types expressed PCV7 serotypes ($n = 32$) and serotype 19A ($n = 10$). Other important serotypes expressed in these clonal types were serotype 15A in 6 strains belonging to CC63, serotype 24F in 5 strains belonging to CC230, and serotype 23B in 3 strains belonging to ST2372.

Table 4 shows the STs detected for each serotype.

Discussion

Based on the results of this study, nine years after the licensing of PCV7 in Spain, the serotypes included in this vaccine are in a minority in our population, especially in children, the target group for the vaccine. Of note, PCV7 serotypes had originally been designed as 'paediatric serotypes', but at present PCV7 serotypes are more frequently detected in adults than children. These data confirm, as have many other studies, that PCV7 has been highly effective against invasive pneumococcal disease (IPD) caused by vaccine serotypes. However, the study also confirms and



Other serotypes in adults 18-64 years: 11A, 11F,15B,18C,23F,35B,6A,6B (2 isolates each) and 13,17F,20,23B,29,33F,38,45 (1 isolate each)
Other serotypes in adults >64 years: 13,15A,17F,18C,19F,24F,29,38,4,6B,9V (2isolates each) and 10B,11,15B,16A,20,25,33F,35F,6C (1 isolate each)

Figure 1 Serotype distribution among 609 isolates of patients with invasive pneumococcal disease according to age group.

Table 3 Percentage of *S.pneumoniae* invasive isolates sensitive to antimicrobial drugs according to age group using Clinical Laboratory Standards Institute criteria.

Percentage of susceptible strains					
Range Age	Penicillin[a]	Cefotaxime[a]	Erythromycin	Tetracycline	Levofloxacin
	MIC ≤.06 mg/L	MIC ≤.5 mg/L	MIC ≤.25 mg/L	MIC ≤2 mg/L	MIC ≤1 mg/L
<2 years	63.4	83.1	69	65.7	100
2–4 years	91.5	94.9	84.7	84.7	100
5–17 years	91.3	97.8	89.1	82.6	100
18–64 years	86.3	93.6	89.5	86.6	97.6
>64 years	76.3	86.7	75.4	77.3	99.5
P[b]	<.001	.012	<.001	.002	NS

[a] Meningeal breakpoints were used for isolates from all types of sterile sites.
[b] Comparison of susceptible strains between age groups.

further reveals the degree to which non-vaccine PCV7 serotypes have occupied the ecological niche left vacant by vaccine serotypes, leading to the emergence of IPD from non-vaccine serotypes. This replacement phenomenon had been reported in two prospective surveillance studies performed in children[14] and adults[15] from two medical centers in Barcelona that found an emergence of IPD caused by non-vaccine serotypes during the vaccination period.

The replacement phenomenon by non-vaccine serotypes is a multifactor event. Great disparity has been reported in the magnitude of replacement around the world, including closed geographical areas. Spain was one of the first geographical areas to suffer this unwanted occurrence. The high intensity of replacement in Spain was caused in part by the introduction of a virulent clone of serotype 1 (not included in PCV7) just before the implementation of PCV7 in our country. In the year 2010, the new conjugate vaccines PCV10 and PCV13 were introduced in Spain, so it is important to clearly characterize the baseline serotypes and clones before the introduction of these vaccines.

According to our results, the potential coverage of PCV10 is limited because multi-resistant clones expressing serotype 19A (which is not included in PCV10) are an important cause of IPD in all age ranges. Moreover, some of these clones such us ST320 or CC230 expressing serotype 19A have been reported in several countries, with great concern.[27–31] ST320 was detected among 13% of 19A isolates (19.5% in children) in this study. These rates are much higher than the 1.7% reported by Ardanuy during 1997–2007 or 4% reported by Tarrago during 2000–2008 in our geographical area.[24,32]

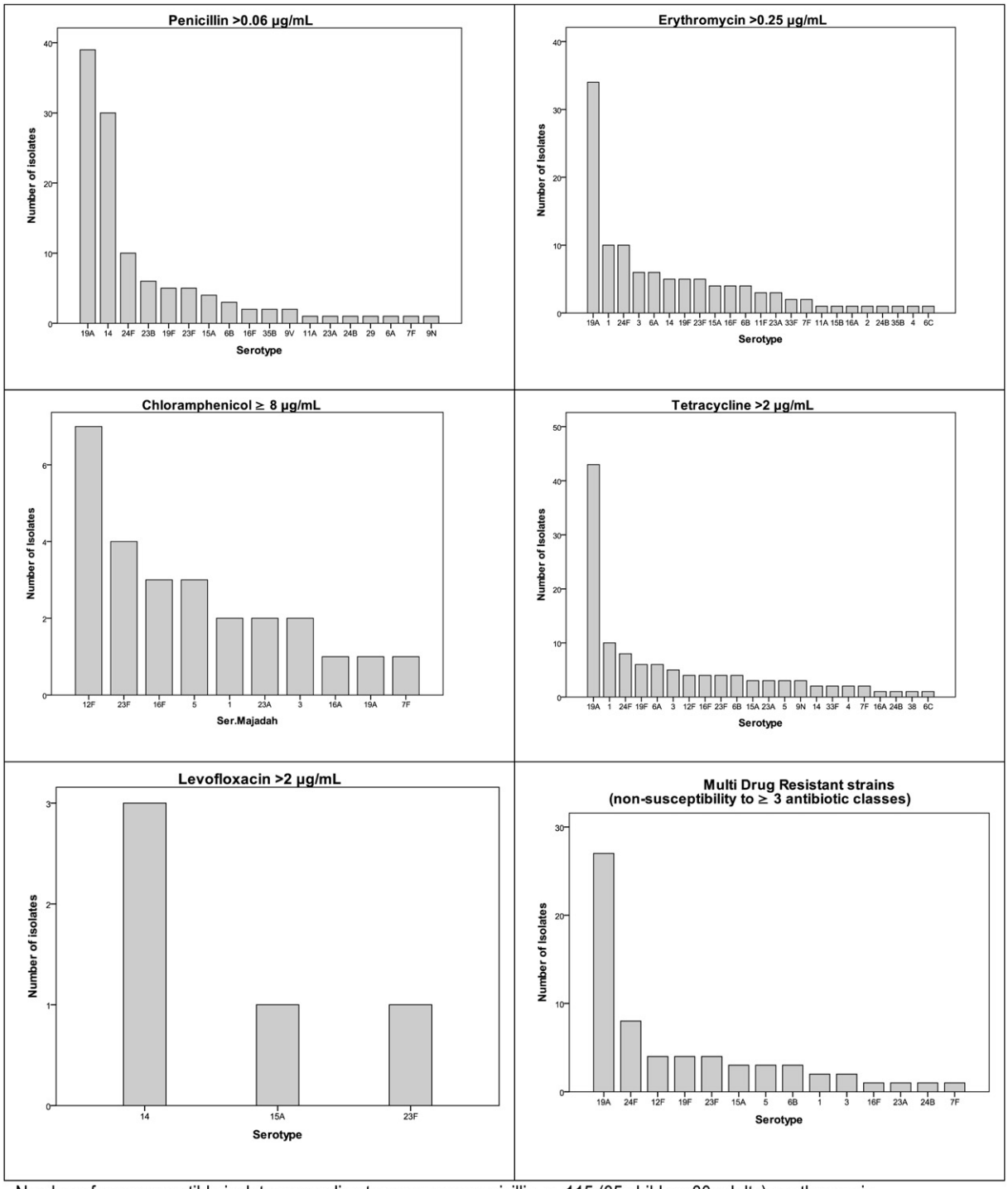
The potential coverage of PCV13 vs PCV10 is significantly much higher in the main group with high incidence of IPD; PCV10 and PCV13 serotypes caused 39.4% and 73.2% of IPD, respectively, in children younger than 24 months of age. The serotype distribution in our paediatric population is quite similar that reported recently by Aguiar et al in Portugal.[33]

At present PCV13 offers good coverage against the main invasive serotypes in all age ranges and remains the best available option to prevent IPD. This high coverage is similar to the rate of 79% found by Picazo et al in children[34] and the 70% and 80.9% found by Grall et al in children and adults from France respectively.[35] The good results against vaccine serotypes obtained with PCV7 might be expected with PCV13. However the replacement phenomenon with non-vaccine serotypes might be also expected. As reported by other authors, some of the main multi-resistant clones detected in our study express non-PCV13 serotypes. Gertz et al[36] found an increase in specifically penicillin non-susceptible clones mainly in serotypes 15A, 35B and 23AC among isolates from the United States, while in our study the main penicillin non-susceptible clones not included in PCV13 were serotypes 24F, 23B and 15A. The clonal study reveals that our emerging clones expressing non-PCV13 serotypes (i.e., ST230 and ST2372) are quite different from those in the American study. Moreover, ST2372 is a novel ST detected for the first time in 2005 in a child from Barcelona with meningitis (www.mlst.net). These geographical differences highlight the importance of local surveillance studies to develop a global picture of the main serotypes and clones around the world.

The evolution and spread of a multi-resistant clone depends mainly on the antibiotic pressure applied in the microbial environment. Our country has a high rate of antibiotic exposure.[37] The emergence of multi-resistant clones that may be detected expressing vaccine serotypes and not vaccine serotypes raises the alarm about the important problem of bad use of antibiotics. Prudent use of antimicrobials in outpatients, inpatients, health-care infections and veterinary use is urgently needed so as to not erode any new vaccine.

Pneumococcal surveillance must also include penicillin-susceptible clones with proven capacity to produce outbreaks. In the past, the presence of ST306 in the first years of implementation of PCV7 vaccine eroded the recognized preventive effect of this vaccine in Spain. In this baseline study we detected ST53 as the main clone found in IPD caused by serotype 8. This clone has been reported as a virulent clone[38] and at present is the dominant clone in serotype 8-related IPD in Scotland.[39] Jefferies et al found a pneumolysin allele 5 in ST53 that could facilitate clonal expansion of serotype 8, which would be of particular concern. In our study ST53 serotype 8 was detected in 6th position in the rank order of the main serotypes in adults less than 64 years old, but it was not detected in children.

Our study shows the high genetic diversity of pneumococci including a large number of STs not previously reported. Moreover, the results show new capsular and clone



Number of non-susceptible isolates according to range age: penicillin, n=115 (35 children,80 adults); erythromycin, n=111 (36 children, 75 adults); choramphenicol, n=26 (4 children,22 adults); tetracycline, n=118 (41 children,77 adults); levofoxacin, n=5 (5 adults); multi-drug resistant,n=64 (26 children, 38 adults)

Figure 2 Serotype distribution among non-susceptible strains according to antimicrobial agent.

combinations (suggesting a capsular switching) involving non-PCV13 serotypes. Therefore if capsular switching results in the expression of a capsule not covered by a conjugate vaccine, this may entail an increase in the rate of IPD.

This study has limitations. First, we do not have available data on clinical evolution of patients which would be interesting to compare differences in morbidity and mortality between conjugate and non-conjugate vaccine serotypes. Second, the use of PCV10 was authorized in Spain in April 2009. However, the use of this vaccine in our country up to November 2009 was very low due to supply problems from the manufacturer, so we can consider year 2009 as a pre-vaccine year. And third, the study doesn't cover 100% of health centers within Catalonia. However,

Table 4 Distribution of clonal types according to serotypes detected in 609 patients with IPD.

Serotype	Number of episodes	%	ST dominant (*n*; %)	Other ST (n)
1[a,c]	137	22.5	ST306 (106; 77.4)	ST228(2), ST304 (25), ST614(2), ST618(2)
7F[b,c]	76	12.5	ST191 (71; 93.4)	ST62(1), ST3544(1), ST3917(1),ST3974(1), ST5137(1)
19A[c]	68	11.2	ST276 (15; 22)	ST81(1), ST193(3), ST199(2), ST202(5), ST320(9), ST416(1), ST876(1), ST994(2), ST1131(1), ST1167(1), ST1201(11), ST1611(1), ST2013(10), ST2220(1), ST2674(1), ST3259(1), ST4831(1), ST5195(1).
3[c]	38	6.2	ST180 (19; 50)	ST260 (12), ST505 (1), ST1220 (1), ST1377 (3), ST2570 (1), ST4675 (1)
14[a,b,c]	33	5.4	ST156 (21; 63.6)	ST15 (1), ST124(1), ST143(1), ST343(2), ST557(4), ST4830(1), ST5193(2).
5[b,c]	25	4.1	ST1223 (18; 72)	ST289 (7)
6A[c]	18	2.9	ST4310 (3; 16.6)	ST65(1), ST224(3), ST327(1), ST460(2), ST1143(1), ST1150(1), ST1692(2), ST2591(1), ST2611(1), ST3787(1), ST3981(1).
9N	18	3.0	ST66 (6; 33.3)	ST67(5), ST280(1), ST1483(1), ST1684(1), ST3982(3), ST5140(1)
10A	14	2.3	ST97 (11; 78.6)	ST1551(1), ST3754(1), ST4309(1)
8	14	2.3	ST53 (7; 50)	ST404(4), ST1012(1), ST1110(1), ST1629(1).
16F	12	2.0	ST30 (7; 58.3)	ST570(2), ST2685(1), ST4022(1), ST5136(1).ç
24F	12	2.0	ST230 (7; 58.3)	ST72(2), ST4677(3)
12F	11	1.8	ST989 (8; 72.7)	ST218(1), ST4833(2)
22F	9	1.3	ST433 (8; 88.8)	ST5138(1)
23F[a,b,c]	9	1.5	ST81 (3; 33.3)	ST36(1), ST85(1), ST109(2), ST277(1), ST1064(1)
4[a,b,c]	8	1.5	ST205 (3; 37.5)	ST246(1), ST1729(1), ST2333(1), ST3254(1), ST4829(1).
19F[a,b,c]	7	1.1	ST2100 (2; 28.6)	ST1(1), ST179(1), ST4307(1), ST4311(1), ST5194(1)
23B	7	1.1	ST2372 (6; 85.7)	ST439(1)
31	7	1.1	ST1684 (3; 42.8)	ST1111(1), ST1766(3)
38	7	1.1	ST393 (4; 57.1)	ST63(1), ST310(2)
11A	6	1.0	ST62 (5; 83.3)	ST4678(1)
18C[a,b,c]	6	1.0	ST113 (2; 33.3)	ST110(1), ST116(1), ST241(1), ST496(1).
6B[a,b,c]	6	1.0	ST1624 (2; 33.3)	ST176(1), ST273(1), ST386(1), ST5222(1)
9V[a,b,c]	6	1.0	ST4306 (2; 33.3)	ST239(1), ST280(1), ST838(1), ST4796(1)
6C	5	8	ST224 (4; 80)	ST4310(1)
11F	5	8	ST62 (4; 80)	ST4674(1)

(*continued on next page*)

Table 4 (*continued*)

Serotype	Number of episodes	%	ST dominant (*n*; %)	Other ST (n)
15A	5	.8	ST63 (3; 60)	ST2613(1), ST5139(1)
23A	5	.8	ST42 (3; 60)	ST38(1), ST2670(1)
35B	5	.8	ST198 (3; 60)	ST373(1), ST558(1)
15B	4	.7	ST101 (2; 50)	ST433(1), ST4828(1)
13	3	.5	ST738 (1; 33.3)	ST923(1), ST2658(1)
17F	3	.5	ST392 (3; 100)	
29	3	.5	ST198 (1; 33.3)	ST558(1), ST2690(1)
20	2	.3	ST1026 (1; 50)	ST1794(1)
27	2	.3	ST4676 (1; 50)	ST1475(1)
33F	2	.3	ST4668 (1; 50)	ST717(1)
10B	1	.2	ST598 (1; 100)	
11	1	.2	ST4305 (1; 100)	
16A	1	.2	ST30 (1; 100)	
2	1	.2	ST1504 (1; 100)	
21	1	.2	ST97 (1; 100)	
24B	1	.2	ST230 (1; 100)	
25	1	.2	ST393 (1; 100)	
28	1	.2	ST433 (1; 100)	
34	1	.2	ST4832 (1; 100)	
35F	1	.2	ST2217 (1; 100)	
45	1	.2	N.A	
Overall	609	100		

Serotypes included in the formulation of PCV7.(a),PCV10 (b) and PCV13 (c)conjugate vaccines.
N.A.: Not available.

the inclusion of 30 important health centers throughout the country may be an accurate representation of total population.

In conclusion, at present PCV13 conjugate vaccine offers good coverage against the main pneumococci serotypes causing IPD in Spain. However, the high genetic diversity of the pneumococcal population highlights the importance of molecular surveillance systems for monitoring IPD during the vaccination period.

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Conflict of interest

Francesc Marco has received honoraria for speaking at scientific meetings sponsored by Novartis. Other authors declare no conflict of interest.

Declaration/responsibility

This paper has neither been published nor submitted for publication elsewhere.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2011.06.002.

References

1. O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 2009;**374**:893—902.
2. Preventpneumo. *Raising the profile of pneumococcal disease*. last accesed 14, http://www.preventpneumo.org/results/pneumoadip_activities/upload/Five-Yr-Media-Report-2003-to-2008-FINAL.pdfData; April 2010.
3. Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Active bacterial core surveillance/emerging infections program network. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* 2010;**201**:32—41.
4. Lucero MG, Dulalia VE, Nillos LT. Pneumococcal conjugate vaccines for preventing vaccine-type invasive pneumococcal disease and X-ray defined pneumonia in children less than two years of age. *Cochrane Database Syst Rev* 2009;**4**:CD004977.
5. Pulido M, Sorvillo F. Declining invasive pneumococcal disease mortality in the United States, 1990—2005. *Vaccine* 2010;**28**:889—92.
6. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. *Clin Infect Dis* 2000;**30**:100—21.
7. Vallès X, Flannery B, Roca A, Mandomando I, Sigaúque B, Sanz S, et al. Serotype distribution and antibiotic susceptibility of invasive and nasopharyngeal isolates of *Streptococcus pneumoniae* among children in rural Mozambique. *Trop Med Int Health* 2006;**11**:358—66.
8. Grijalva CG, Nuorti JP, Arbogast PG, Martin SW, Edwards KM, Griffin MR. Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis. *Lancet* 2007;**369**:1179—86.
9. De Wals P, Robin E, Fortin E, Thibeault R, Ouakki M, Douville-Fradet M. Pneumonia after implementation of the pneumococcal conjugate vaccine program in the province of Quebec, Canada. *Pediatr Infect Dis J* 2008 Nov;**27**(11):963—8.
10. Jardine A, Menzies RI, McIntyre PB. Reduction in hospitalizations for pneumonia associated with the introduction of a pneumococcal conjugate vaccination schedule without a booster dose in Australia. *Pediatr Infect Dis J* 2010 Jul;**29**(7):607—12.
11. Li ST, Tancredi DJ. Empyema hospitalizations increased in US children despite pneumococcal conjugate vaccine. *Pediatrics* 2010 Jan;**125**(1):26—33. 30.
12. Eastham KM, Freeman R, Kearns AM, Eltringham G, Clark J, Leeming J, et al. Clinical features, aetiology and outcome of empyema in children in the north east of England. *Thorax* 2004;**59**:522—5.
13. Byington CL, Samore M, Stoddard GJ, Barlow S, Daly J, Korgenski K, et al. Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of nonvaccine serogroups. *Clin Infect Dis* 2005;**41**:21—9.
14. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. Emergence of invasive pneumococcal disease caused by non-vaccine serotypes in the era of 7-valent conjugate vaccine. *Clin Infect Dis* 2008;**46**:174—82.
15. Ardanuy C, Tubau F, Pallares R, Calatayud L, Domínguez MA, Rolo D, et al. Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997—2007. *Clin Infect Dis* 2009;**48**:57—64.
16. Lin CJ, Chen PY, Huang FL, Lee T, Chi CS, Lin CY. Radiographic, clinical, and prognostic features of complicated and uncomplicated community-acquired lobar pneumonia in children. *J Microbiol Immunol Infect* 2006;**39**:489—95.
17. Greenberg D, Givon-Lavi N, Newman N, Bar-Ziv J, Dagan R. Nasopharyngeal carriage of individual Streptococcus pneumoniae serotypes during pediatric pneumonia as a means to estimate serotype disease potential. *Pediatr Infect Dis J* 2011 Mar;**30**(3):227—33.
18. Jefferies JM, Smith AJ, Edwards GF, McMenamin J, Mitchell TJ, Clarke SC. Temporal analysis of invasive pneumococcal clones from Scotland illustrates fluctuations in diversity of serotype and genotype in the absence of pneumococcal conjugate vaccine. *J Clin Microbiol* 2010 Jan;**48**(1):87—96.
19. Brueggemann AB, Spratt BG. Geographic distribution and clonal diversity of Streptococcus pneumoniae serotype 1 isolates. *J Clin Microbiol* 2003;**41**:4966—70.
20. Informe del registre del conjunt minim Basic de dades (CMBD). *Servei Catala de la Salut*. Available at, http://www10.gencat.cat/catsalut/cat/prov_cmbd.htmData; June 9, 2010. last accessed.
21. Tarrago D, Fenoll A, Sanchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. *Clin Microbiol Infect* 2008;**14**:828—34.
22. National Committee for Clinical Laboratory Standards (NCCLS). *Performance standards for antimicrobial susceptibility testing. Eighteenth informational supplement. CLSI document M100—S18*. ISBN 1-5-56238-653-0. Wayne PA: Clinical and Laboratory Standard Institute; 2008.
23. Muñoz-Almagro C, Esteva C, de Sevilla MF, Selva L, Gene A, Pallares R. Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. *J Infect* 2009 Aug;**59**(2):75—82.
24. Ardanuy C, Rolo D, Fenoll A, Tarrago D, Calatayud L, Liñares J. Emergence of a multidrug-resistant clone (ST320) among invasive serotype 19A pneumococci in Spain. *J Antimicrob Chemother* 2009 Sep;**64**(3):507—10.
25. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* 1998;**144**:3049—60.
26. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004;**186**:1518—30.
27. Choi EH, Kim SH, Eun BW, Kim SJ, Kim NH, Lee J, et al. *Streptococcus pneumoniae* serotype 19A in children, South Korea. *Emerg Infect Dis* 2008;**14**:275—81.
28. Pillai DR, Shahinas D, Buzina A, Pollock RA, Lau R, Khairnar K, et al. Genome-wide dissection of globally emergent multi-drug resistant serotype 19A *Streptococcus pneumoniae*. *BMC Genomics* 2009;**10**:642.
29. Aguiar SI, Pinto FR, Nunes S, Serrano I, Melo-Cristino J, Sá-Leão R, et al. Denmark 14-230 clone as an increasing cause

of pneumococcal infection in Portugal within a background of diverse serotype 19A lineages. *J Clin Microbiol* 2010;**48**:101—8.
30. Sener B, McGee L, Pinar A, Eser O. Genomic backgrounds of drug-resistant *streptococcus pneumoniae* in Ankara, Turkey: identification of emerging new clones. *Microb Drug Resist* 2006;**12**:109—14.
31. Moore MR, Gertz Jr RE, Woodbury RL, Barkocy-Gallagher GA, Schaffner W, Lexau C, et al. Population snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States, 2005. *J Infect Dis* 2008;**197**:1016—27.
32. Tarragó D, Aguilar L, García R, Gimenez MJ, Granizo JJ, Fenoll A. Evolution of clonal and susceptibility profiles of serotype 19A Streptococcus pneumoniae among invasive isolates from children in Spain, 1990 to 2008. *Antimicrob Agents Chemother* 2011 May;**55**(5):2297—302.
33. Aguiar SI, Brito MJ, Gonçalo-Marques J, Melo-Cristino J, Ramirez M. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. *Vaccine* 2010; **28**:5167—73.
34. Picazo J, Ruiz-Contreras J, Casado-Flores J, Giangaspro E, Del Castillo F, Hernández-Sampelayo T, et al. Heracles Study Group. Relationship between serotypes, age, and clinical presentation of invasive pneumococcal disease in Madrid, Spain, after introduction of the 7-valent pneumococcal conjugate vaccine into the vaccination calendar. *Clin Vaccine Immunol* Jan 2011;**18**(1):89—94.
35. Grall N, Hurmic O, Al Nakib M, Longo M, Poyart C, Ploy MC, et al. ORP Ile de France Ouest. Epidemiology of Streptococcus pneumoniae in France before introduction of the PCV-13 vaccine. *Eur J Clin Microbiol Infect Dis*; Apr 18 2011 [Epub ahead of print].
36. Gertz RE, Li Z, Pimenta FC, Jackson D, Juni BA, Lynfield R, et al. Active bacterial core surveillance team. Increased penicillin nonsusceptibility of nonvaccine-serotype invasive pneumococci other than serotypes 19A and 6A in post-7-valent conjugate vaccine era. *J Infect Dis* 2010;**201**:770—5.
37. Special eurobarometro 338. *Antimicrobial resistance*. Available from, http://ec.europa.eu/health/antimicrobial_resistance/docs/ebs_338_en.pdfData; November-December 2009. last accessed 14 may 2011.
38. Birtles A, McCarthy N, Sheppard CL, Rutter H, Guiver M, Haworth E, et al. Multilocus sequence typing directly on DNA from clinical samples and a cultured isolate to investigate linked fatal pneumococcal disease in residents of a shelter for homeless men. *J Clin Microbiol* 2005;**43**:2004—8.
39. Jefferies JM, Johnston CH, Kirkham LA, Cowan GJ, Ross KS, Smith A, et al. Presence of nonhemolytic pneumolysin in serotypes of *streptococcus pneumoniae* associated with disease outbreaks. *J Infect Dis* 2007;**196**:936—44.

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ARTICLE

DNA bacterial load in children and adolescents with pneumococcal pneumonia and empyema

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Abstract The purpose of this investigation was to evaluate a rapid quantitative real-time polymerase chain reaction (PCR) for the direct detection and quantification of pneumococcal DNA bacterial load (DBL) in patients with pneumonia and empyema. DBL and molecular serotype detection was determined by DNA quantification of the pneumolysin (*ply*) gene and an additional capsular gene by real-time PCR. Plasma or pleural fluid samples from children and adolescents with confirmed pneumococcal pneumonia were analyzed. DBL was correlated with clinical parameters and outcomes. One hundred and sixty-nine patients with pneumococcal pneumonia (145 empyema) had bacterial cultures and real-time PCR assays performed. Among them, 41 (24.3%) had positive results for both, 4 (2.4%) had positive culture alone, and 124 (73.3%) had positive real-time PCR alone. The pleural fluid DBL was lower in patients with prior antibiotics ($p=0.01$) and higher in patients with positive culture ($p<0.001$). The pleural fluid DBL was positively correlated with serum C-reactive protein ($p=0.009$), pleural fluid neutrophils ($p<0.001$), and pleural fluid glucose ($p<0.001$). The plasma and pleural fluid DBL were higher in patients with ≥8 days of hospital stay ($p=0.002$), and the pleural fluid DBL was positively correlated with the number of hours of pleural drainage ($p<0.001$). Quantification of pneumococcal DBL by real-time PCR may be helpful for the diagnosis and clinical management of pediatric patients with pneumonia and empyema

Declaration/responsibility: this paper has neither been published nor submitted for publication elsewhere.

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Introduction

Streptococcus pneumoniae is one of the major bacterial pathogens causing severe infections with high morbidity and mortality [1]. There are 93 pneumococcal capsular types (serotypes), but only a limited number of them cause the majority of invasive pneumococcal disease (IPD) [2].

The gold standard culture-based method for the diagnosis of IPD requires the isolation and identification of *Streptococcus pneumoniae* from normally sterile clinical specimens; it needs up to 48–72 h to confirm the results and may have a low sensitivity [3].

It is well known that many febrile children who are seen at the Emergency Department had received prior antibiotics and it can prevent the growth of *Streptococcus pneumoniae* in cultures [4]. Therefore, several episodes of infection caused by antibiotic-susceptible pneumococcal serotypes may be misdiagnosed and, consequently, the rates of IPD can be underestimated. In order to improve the diagnosis of

IPD and also to perform valuable epidemiological surveillance studies, we need additional methods with higher sensitivity and which can be carried out in a shorter period of time than the standard culture-based method.

Molecular techniques applied to quantify viral load are widely accepted for monitoring the course of infections such as hepatitis C or human immunodeficiency virus (HIV) [5–8]. However, little is known about the usefulness of the direct detection and quantification of pneumococcal DNA in patients with IPD. Current molecular techniques such as pneumococcal real-time polymerase chain reaction (PCR) (direct detection of pneumococcal DNA) may be rapid methods to improve the diagnosis of IPD and also for serotyping. Moreover, real-time PCR may allow determining the quantification of pneumococcal DNA bacterial load (DBL) in different clinical samples and improving the management of patients with IPD, such as those which occur in some viral infections [9, 10]. To our knowledge, there are no reports about the plasma and pleural fluid DBL in children with pneumonia and empyema.

The objectives of this study were: (1) to evaluate a rapid quantitative real-time PCR for the direct detection and quantification of pneumococcal DBL in sterile clinical samples, and (2) to examine the relationship between DBL (in plasma and pleural fluid) with several clinical and microbiological variables in children with pneumonia and empyema.

Patients and methods

We prospectively studied all children and adolescents (less than 18 years of age) with confirmed pneumococcal pneumonia who attended the Sant Joan de Deu Hospital, a 345-bed children's hospital in Barcelona, Spain, from 6/2003 to 6/2008.

Confirmed pneumococcal pneumonia was defined as the presence of clinical and radiological findings of pneumonia, together with the isolation of *Streptococcus pneumoniae* and/or DNA detection of *Streptococcus pneumoniae* by real-time PCR in plasma and/or pleural fluid. Plasma and/or pleural fluid samples were obtained when available and according to patients' symptoms. For the present study, we have selected those children with confirmed pneumococcal pneumonia who had been studied by both culture and real-time PCR.

As a control group, the real-time PCR assay was assessed with 106 plasma samples from healthy individuals, 50 of whom were healthy nasopharyngeal carriers of *Streptococcus pneumoniae*. Blood samples were extracted in the outpatient's clinic prior to a minor surgical procedure (such us ocular surgery, phimosis, or non-complicated hernia) and after written informed consent was obtained.

Microbiological bacterial cultures

All pneumococcal isolates were identified by standard microbiological methods as previously described [11, 12].

Serotype identification

The serotyping of strains isolated by culture was carried out by the Quellung reaction at the National Center for Microbiology (Majadahonda, Madrid, Spain). The detection of pneumococcal serotypes in clinical samples with negative culture were performed in our laboratory according to multiplex real-time PCR [13]. This sequential PCR approach distinguishes among 24 serotypes (1, 3, 4, 5, 6A, 6B, 7F/A, 8, 9V/A/N/L, 14, 15B/C, 18C/B, 19A, 19F/B/C, 23A, and 23F). Serotypes were classified into two groups: PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) and non-PCV7 serotypes including all others.

Extraction of DNA

The DNA of pneumococci in sterile samples was extracted using 20% w/v Chelex-100 resin (BioRad Laboratories, Hercules, CA, USA). Plasma and pleural fluid samples required previous preparation as follows: 200 μl of samples were centrifuged at 4°C and 25,000g for 60 min. After removal of the supernatant, samples were resuspended in 100 μl of PBS buffer. Then, 50 μl of prepared samples were added and vortexed with 150 μl of 20% w/v Chelex-100. The tubes were incubated for 20 min at 56°C followed by a 10-min incubation at 99°C. After cooling, the supernatant was used as a template in the real-time PCR experiments.

DNA from samples collected since 2006 were extracted from 200 μl of biological fluid (plasma, pleural fluid) by an automated system for DNA extraction (easyMAG, bioMérieux Laboratories). Selected samples were performed in parallel in order to compare the recovery of bacterial DNA using both DNA extraction methods.

DNA quantification by the real-time PCR assay

TaqMan fluorescent probes and specific primers for the pneumolysin (*ply*) gene of *Streptococcus pneumoniae* (GenBank accession no. MX52474) [14] that have been previously described by Corless et al. were used [15]. In addition, these primer sets were tested using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA) for universal conditions of amplification. Amplification was done in a real-time PCR instrument ABI PRISM 7000 (Applied Biosystems, Foster City, CA, USA). The reaction volume of 25 μl contained 5 μl of DNA extract from samples or controls and 2× TaqMan

Universal Master Mix (Applied Biosystems), which includes dUTP and uracil-*N*-glycosylase; each primer was used at a final concentration of 300 nM. The TaqMan probe was used at a final concentration of 150 nM. Internal controls for monitoring false-negatives by PCR inhibitors were run for each sample to monitor the overall assay performance, and these consisted of 1 μl of TaqMan RNase P Control Reagent (VIC) (Applied Biosystems, Foster City, CA, USA) that included human RNase P primers and the TaqMan Probe with VIC as the fluorescent reporter dye at the 5′ end. Amplification was performed using universal amplification conditions: incubation for 2 min at 50°C (uracil-*N*-glycosylase digestion) and 10 min of denaturation at 95°C, 45 cycles of two-step amplification (15 s at 95°C and 60 s at 60°C). The amplification data were analyzed by SDS Software (Applied Biosystems). The reporter dye (FAM for samples or VIC for the internal control) signal was measured relative to the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. The cycle threshold (C_T) value was defined as the cycle at which the reporting dye fluorescence first exceeds the calculated background level. A low C_T value thus corresponds to a high target concentration. The quantification of *Streptococcus pneumoniae* in each sample was based on a standard curve generated by plotting the C_T value against known genomic equivalents.

Determination of PCR efficiency and the calibration curve for DNA quantification

A 0.5 McFarland suspension was made from a serotype 19F *Streptococcus pneumoniae* strain obtained from our bacteriological laboratory and serial dilutions from 1.7 ng/μl (7×10^7 CFU/mL) to 0.6 fg/μl (7 CFU/mL) were performed. These serial dilutions were used to generate a reference standard curve which was exported and used as an external standard curve after each run. The external standard curve was calibrated with two standard controls included in each run with the clinical samples. DNA extraction of these serial dilutions was performed by using a manual method and the easyMAG platform.

The reproducibility of triplicate C_T values was assessed on all standard specimens. The mean intra-assay and inter-assay coefficient of variation were calculated from the C_T values.

Statistical analysis

Statistical analysis was performed with the PASW software package (version 17.0). Pneumococcal DBL data were log-transformed to assume a normal distribution. Continuous variables were compared using the *t*-test (for approximately normally distributed data) or the Mann–Whitney *U*-test (for skewed data) and are described as mean values and standard deviations or median and interquartile range (IQR; 25th–75th percentiles) according to the presence of normal distribution, respectively. The Chi-square test or Fisher's exact test (two-tailed) were used to compare categorical variables. Associations between routine biochemical variables and DBL were examined using Spearman's correlation coefficient. Comparison between groups was performed by the Kruskal–Wallis test. Logistic regression models were used to examine the independent effect of DBL on the length of hospital stay after adjusting for other variables. Statistical significance was set at a *p*-value of <0.05.

Results

Overall, there were 206 children with confirmed pneumococcal pneumonia and we included in the study 169 children (145 with empyema and 24 without empyema) who had both bacterial cultures and real-time PCR performed. Of them, 41 (24.3%) had both positive culture and real-time PCR, 4 (2.4%) had positive culture and negative real-time PCR, and 124 (73.3%) had positive real-time PCR and negative culture.

Reproducibility and specificity of the real-time PCR assay

The real-time PCR assay detected correctly all serial dilutions (range 7 to 7×10^7 CFU copies/mL). There was a linear correlation between the log of the standards and the threshold cycles (C_T), with a regression line showing a slope of −3.5 and a Pearson correlation coefficient of 0.99 when DNA extraction was made by the Chelex-100 method or by the easyMAG method. The amount of PCR product formed was derived from the function $y=-3.5x+29.4$ for the Chelex method and $y=-3.5x+32.2$ for easyMAG method. The reproducibility was calculated using the standard curve that was amplified in three consecutive runs using the manual method and the easyMAG method. The standard deviation of the mean C_T was always less than one cycle for the two procedures.

The real-time PCR was highly specific; only two of the 106 plasma samples from healthy children were positive by real-time PCR, and they had a low pneumococcal DBL (5 and 2 CFU copies/mL, respectively). Of note, one of these two children was a pneumococcal nasopharyngeal carrier and the other was, presumably, a non-identified carrier.

Pneumococcal DBL and clinical characteristics

There were 165 children with pneumonia and positive detection of pneumococcal DNA in sterile samples (one or

more). Pleural fluid was positive in 126 of 129 samples collected (97.6%) and plasma was positive in 85 of 137 (62%) samples. Among 145 children with empyema, plasma and pleural fluid were analyzed in 101, and 52 (51.5%) were positive for pleural fluid only, 3 (3%) were positive for plasma only, and 46 (45.5%) were positive for both.

Overall, there were 89 males (53.9%), and the mean age was 54.2 months (standard deviation [SD] 38.8 months) (range 1 month to 17 years). According to the criteria of the American Academy of Pediatrics [16], 9 patients (6.2%) had risk factors for IPD as follows: chronic pulmonary disease 6, chronic cardiac disease 2, and measles coinfection 1. Prior vaccination with PCV7 was detected in 42 of 150 patients (28%) who had available information, and all of them were infected by non-vaccine serotypes. The mean number of hours of fever before admission (in 161 cases) were 107.5 hours (SD 64 hours). Prior antibiotics were identified in 84 of 153 patients (54.9%) who had available information. Of the 165 pneumococcal pneumonia cases, 126 were serotyped (76.3%) (41 by conventional serotyping in culture-positive samples and 85 by multiplex real-time PCR in culture-negative samples); sufficient material for serotyping was not available in 39 patients. Of the 126 episodes with serotype information, 10 (7.9%) were caused by vaccine serotypes and 116 (92.1%) by non-vaccine serotypes. The main serotypes detected were serotype 1 (53 patients; 42%), serotype 19A (16 patients; 12%) , serotype 3 (11 patients; 8.7%), 7F (11 patients; 8.7%), and serotype 5 (6 patients; 4.7%). Fourteen of 165 patients (8.5%) were admitted to the pediatric intensive care unit (PICU). The mean length of hospital stay was 11.4 days (SD 6.5 days). None of the patients died.

As shown in Fig. 1, the pneumococcal DBL was much higher in pleural fluid than in plasma (Mann–Whitney test, $p<0.001$). According to the clinical syndrome, the plasma DBL was slightly higher in patients with pneumonia and empyema ($n=65$) than in patients with non-complicated pneumonia (without empyema) ($n=20$): bacterial load median $\log_{10}$ CFU copies/mL (IQR): 1.14 (0.44–1.59) versus 0.97 (0.55–1.15), but it did not reach statistical significance (Mann–Whitney test, $p=0.4$).

Table 1 shows the plasma and pleural fluid DBL according to several parameters. Most clinical and microbiological variables were not associated with differences in the plasma DBL. However, the plasma DBL was significantly higher in patients with ≥8 days of hospital stay as compared with those with <8 days of stay (Table 1).

As shown in Table 1, the pleural fluid DBL was lower in patients with prior antibiotic therapy (as compared with no prior antibiotics) ($p=0.01$), and was higher in those with positive culture for *Streptococcus pneumoniae* (as compared with negative cultures) ($p<0.001$). In addition, as

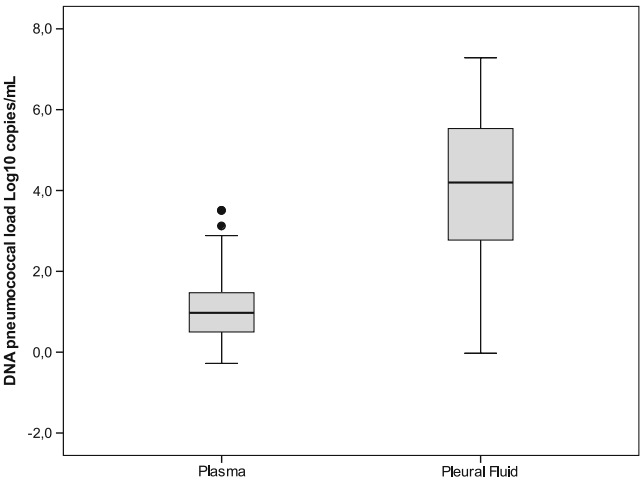

Fig. 1 Pneumococcal DNA bacterial load (DBL) in plasma and pleural fluid samples. Plasma; $n=85$, DBL median $\log_{10}$ CFU copies/mL (interquartile range [IQR]): 1.10 (0.50–1.47). Pleural fluid; $n=126$, DBL median $\log_{10}$ CFU copies/mL (IQR): 4.07 (2.76–5.53). Mann–Whitney test DNA pneumococcal load in plasma versus pleural fluid, $p<0.001$

occurred in plasma DBL, there were higher levels of pleural fluid DBL in patients with ≥8 days of hospital stay as compared with those with ≤7 days. Using multiple logistic regression models, we found that the plasma DBL (adjusted odds ratio [OR] 3.53; 95% confidence interval [CI]: 1.43–8.70; $p=0.006$) and the pleural fluid DBL (adjusted OR 1.46; 95% CI: 1.09–1.96; $p=0.01$) were independently associated with prolonged hospital stay (≥8 days) after adjusting for age, hours of fever before admission, and positive bacterial culture.

Figure 2 shows a positive correlation between the pleural fluid DBL and the number of days spent in the hospital (Spearman's rho: 0.4; $p<0.001$) and the total number of hours of pleural drainage (Spearman's rho: 0.4; $p<0.001$).

As shown in Fig. 3, the pleural fluid DBL showed a positive correlation with the plasma DBL (Spearman's rho: 0.3; $p<0.01$). In addition, the pleural fluid DBL had a significant correlation with several biochemical markers of infection, such as serum C-reactive protein (CRP) (Spearman's rho: 0.2 ; $p=0.009$), pleural fluid neutrophils (Spearman's rho: 0.4; $p<0.001$), and pleural fluid glucose levels (Spearman's rho: −0.8; $p<0.001$).

Discussion

DNA detection and quantification may provide useful epidemiological and clinical information and improve our knowledge about the dynamics of pneumococcal replication. Two major findings are highlighted in this study. First, the significant increase of detecting pneumococcal pneumonia and empyema in children and subsequent serotyping

Table 1 Plasma and pleural fluid pneumococcal DNA bacterial load (DBL) according to different parameters in children with pneumonia

	Plasma DBL			Pleural fluid DBL		
	No. of patients studied	Median $\log_{10}$ CFU copies/mL (IQR)	*p*-value	No. of patients studied	Median $\log_{10}$ CFU copies/mL (IQR)	*p*-value
Sex						
Male	49	1.15 (0.67–1.53)	0.4	63	4.01 (2.75–5.35)	0.7
Female	36	1.03 (0.43–1.47)		63	4.04 (3.05–5.60)	
Age group						
0–23 months	18	1.22 (0.64–2.02)	0.4	21	4.20 (3.13–5.45)	0.7
≥24 months	67	1.07 (0.48–1.40)		105	4.04 (2.69–5.61)	
PCV7 vaccination						
Yes	21	1.02 (0.37–1.83)	0.7	35	3.94 (2.74–5.22)	0.7
No	55	1.09 (0.59–1.43)		80	4.04 (2.76–5.65)	
Prior antibiotic exposure						
Yes	42	1.25 (0.68–1.74)	0.1	68	3.71 (2.61–5.00)	0.01
No	36	0.94 (0.41–1.20)		51	4.52 (3.08–6.31)	
Hours of fever before admission						
0–72 h	28	1.00 (0.48–1.26)	0.4	40	3.86 (2.21–5.47)	0.3
>72 h	57	1.15 (0.49–1.59)		86	4.17 (2.87–5.54)	
Presence of underlying diseases (risk factors)						
Yes	5	1.04 (0.28–1.26)	0.8	4	3.50 (0.75–5.96)	0.5
No	80	1.11 (0.51–1.47)		122	4.09 (2.82–5.53)	
Microbiological data						
Positive culture	25	1.11 (0.48–1.80)	0.9	36	5.57 (5.18–6.39)	<0.001
Negative culture	60	1.10 (0.55–1.45)		90	3.47 (2.04–4.78)	
PCV7 serotypes	8	1.64 (0.97–2.08)	0.1	7	4.46 (3.61–5.82)	0.9
Non-PCV7 serotypes	60	1.12 (0.45–1.59)		92	4.41 (3.34–5.70)	
Outcomes						
PICU admission						
Yes	7	1.54 (0.71–2.17)	0.3	12	4.04 (2.59–5.68)	0.9
No	78	1.06 (0.46–1.44)		114	4.07 (2.76–5.52)	
Days of hospital stay						
0–7 days	28	0.78 (0.38–0.99)	0.002	29	3.20 (1.85–4.13)	0.002
≥8 days	57	1.28 (0.71–1.76)		97	4.33 (3.39–5.61)	

DBL in plasma was studied in 85 of the 165 patients with pneumonia with and without empyema and DBL in pleural fluid was studied in 126 of the 145 patients with empyema

IQR, interquartile range; the Mann–Whitney *U*-test was used for skewed data and unpaired Student's *t*-test was used for approximately normally distributed data

when adding real-time PCR for diagnosis to traditional culture-based methods. And second, a positive correlation of pleural fluid pneumococcal DBL with several clinical and outcome variables.

To date, the reported studies on the use of PCR for the diagnosis of IPD have shown controversial results. Recently, Avni et al. [17] published an accurate meta-analysis and found that the studies were highly heterogeneously including different types of PCR methods [18, 19], different target genes [20], most used stored frozen [21] and different samples (i.e., whole blood or plasma) [22], as well as different patient characteristics and different clinical syndromes [23, 24]. The authors concluded that "the lack of appropriate reference standard might have caused underestimation of the performance of the PCR" and that "currently available methods for PCR with blood samples for the diagnosis of IPD lack the sensitivity and specificity necessary for clinical practice."

In our study, we used fresh plasma and pleural fluid samples for the detection and quantification of the DNA and, in most cases, we have added the identification of serotypes by a multiplex real-time PCR, which allowed us

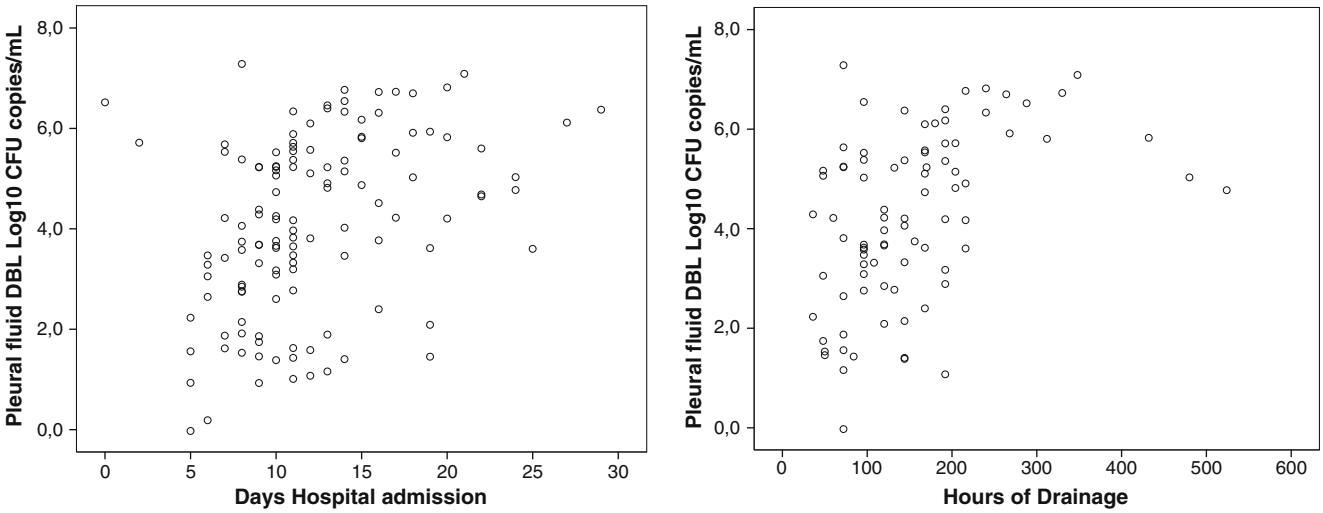

Fig. 2 Pneumococcal DBL in pleural fluid samples in relation to outcomes. Outcomes: pleural fluid DBL and days of hospital stay, Spearman's rho: 0.4; $p<0.001$; pleural fluid DBL and hours of pleural drainage, Spearman's rho: 0.4; $p<0.001$

to increase significantly the number of confirmed episodes with respect to the culture-based method, as occurred in the study reported by Azzari et al. [19]. Moreover, in the meta-analysis by Avni et al. [17], the rate of diagnosis by positive PCR was also higher than the rate by positive blood culture in 16 of 25 studies.

Some authors have warned of reporting IPD by PCR only because of the possibility of detecting false-positive results in blood samples of healthy children [25]. In our study, we used plasma samples and found a good specificity (only two samples of 106 tested from healthy controls). Of note, the worst specificity was found when using buffy coat as the blood sample [26] instead of plasma samples. One limitation of our study is that we used the *ply* gene for the detection (diagnosis) and quantification of pneumococcal DBL. The *ply* gene may be unspecific because some other strains closely related with *Streptococcus pneumoniae* may share the virulent genes encoding by *Streptococcus pneumoniae*, such as *ply* or *lytA* genes [27]. Carvalho Mda et al. [20] tested two *ply*-based PCR assays against 10 *Streptococcus pseudopneumoniae* isolates and 11 isolates of pneumococcus-like viridans group streptococci (P-LVS) and found a positive reaction in all of them. However, we may consider that, in our study, the consecutive serotyping by real-time PCR (which included testing with capsular genes of pneumococci) and the detection of a virulent gene (the *ply* gene) of pneumococci in sterile samples of patients with clinical symptoms and radiologically confirmed pneumonia and empyema may have significant clinical value. To date, the clinical role of *Streptococcus pseudopneumoniae* and other quasi-pneumococcus in invasive disease is unknown, but some authors have warned about the increase of these strains in the nasopharynx [28]. So, the clinical role of these closely related pneumococcal strains isolated in sterile samples need to be clarified.

In our study, the pleural fluid DBL was higher than the plasma DBL, and there was a positive correlation between both of them. Plasma DBL levels were not significantly associated with age, sex, underlying conditions, and other clinical and microbiological variables, but was higher in children who had ≥8 days of hospital stay (Table 1). A low level of plasma DBL was also found in other studies performed by culture-based methods [29] or by real-time PCR [30, 31], and this may explain, at least in part, the low rate of positive blood cultures detected and the poor correlation of blood culture results with DBL (the plasma DBL was similar in children with positive and negative blood cultures). It is probably that the main factor for detecting pneumococci in blood culture-based methods (or by PCR-based methods) is related to the volume of sample processed, and in younger children, the blood volume is usually very low (approximately 1–2 ml in children less than 6 months of age or 2–5 ml in children between 6 months and 5 years of age). On the contrary, the pleural fluid DBL was highly correlated with the pleural fluid culture results (the pleural fluid DBL was significantly higher in cases with positive pleural fluid culture than in negative samples). It may be related to the inoculated volume for pleural fluid culture (approximately 5–10 ml of pleural fluid sample), which is usually higher than in blood cultures. However, the pleural fluid DBL levels were still high in patients with negative pleural fluid culture, and this may suggest that other factors should explain the low rate of positive culture in this type of sample. One may speculate that pleural fluid samples are usually sent to the laboratory in a sterile tube without transport medium, and this may be critical to reach a viable number of bacteria for culture. Our data

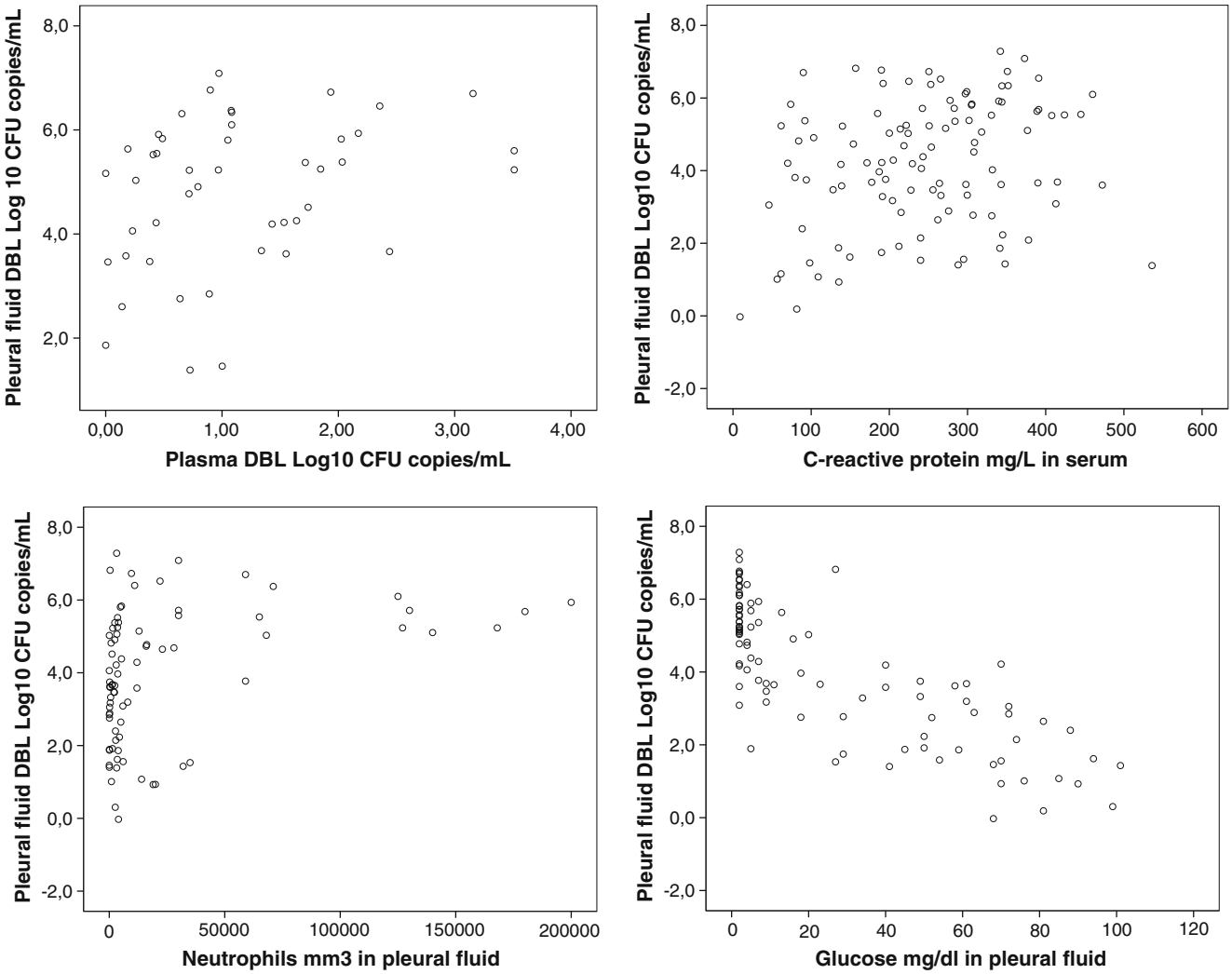

Fig. 3 Pneumococcal DBL in pleural fluid samples in relation to the plasma DBL, and routine hematological and biochemical markers. Pleural fluid DBL and plasma DBL., Spearman's rho: 0.3; $p=0.01$. Pleural fluid DBL and C-reactive protein in serum, Spearman's rho: 0.2; $p=0.009$. Pleural fluid DBL and neutrophils counts in pleural fluid, Spearman's rho: 0.4; $p<0.001$. Pleural fluid DBL and glucose levels in pleural fluid, Spearman's rho: −0.8; $p<0.001$

suggest the need for improving the transport of pleural fluid samples for bacteriological culture.

Some studies have reported that prior antibiotics may affect the sensitivity of PCR assays [23, 32], while others did not [22, 33]. In our study, we have found that the plasma DBL was very low and not significantly different between those with prior and no prior antibiotics, but the pleural fluid DBL was higher in those with no prior antibiotics (Table 1). There were no significant differences in the plasma and pleural DBL levels between patients infected with PCV7 serotypes versus non-vaccine serotypes. However, it should be noted that several serotypes are included in each group and the low number of each specific serotype precluded further analyses. Positive correlations were observed for the pleural fluid DBL with plasma DBL, as well as with several routine hematological and biochemical parameters, such as serum CRP, pleural fluid neutrophils, and pleural fluid glucose levels.

Regarding the outcomes, both the plasma and pleural fluid DBL levels were higher in patients who had ≥8 days of hospital stay (Table 1). We could not correlate the DBL with mortality because none of our children died. Our data are in accordance with previous reports showing a relationship between DBL and worse outcome [10, 34] and prolonged length of hospital stay [9] in patients with severe pneumococcal infections, such as pneumonia and meningitis; as well as that occurred in patients with meningococcal meningitis in whom there was an association between bacterial load and mortality, complications, sequelae, and length of hospital stay [35].

We found that, in our study children, the pleural fluid DBL was correlated with the number of hours of drainage

(length of pleural drainage) (Fig. 1). The treatment of pneumococcal empyema in children may be controversial, and recent studies have suggested that an early use of surgery with video-assisted thoracoscopy is associated with lower in-hospital mortality, length of hospital stay, and duration of antibiotic therapy, compared with a non-surgical group [36]. However, a pediatric surgeon may not always be available in all hospitals. These data suggest that the pleural fluid DBL (high levels) could help in the management of children with pleural pneumococcal empyema, and further studies are needed to evaluate this hypothesis.

In conclusion, the quantification of pneumococcal DBL by real-time PCR may be helpful for the diagnosis and clinical management of pediatric patients with pneumonia and empyema.

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Potential conflict of interest: D.T. has been an advisor for GlaxoSmithKline Biologicals and Wyeth Vaccines. All other authors: no conflict of interest.

References

1. World Health Organization (WHO) (2003) Pneumococcal vaccines. Wkly Epidemiol Rec 14:110–119
2. Hausdorff WP, Bryant J, Paradiso PR, Siber GR (2000) Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I. Clin Infect Dis 30:100–121
3. Gillespie SH (1999) The role of the molecular laboratory in the investigation of *Streptococcus pneumoniae* infections. Semin Respir Infect 14(3):269–275
4. Clements H, Stephenson TJ (1996) Blood culture is poor method of confirming pneumococcus as cause of childhood pneumonia. BMJ 313:757
5. Funk GA, Gosert R, Hirsch HH (2007) Viral dynamics in transplant patients: implications for disease. Lancet Infect Dis 7:460–472
6. Chevaliez S, Pawlotsky JM (2005) Use of virologic assays in the diagnosis and management of hepatitis C virus infection. Clin Liver Dis 9:371–382
7. Young B, Kuritzkes DR (1999) Viral kinetics: implications for treatment. AIDS 13(Suppl 1):S11–S17
8. Riddler SA, Mellors JW (1997–1998) HIV-1 viral dynamics and viral load measurement: implications for therapy. AIDS Clin Rev 47–65
9. Peters RP, de Boer RF, Schuurman T, Gierveld S, Kooistra-Smid M, van Agtmael MA et al (2009) *Streptococcus pneumoniae* DNA load in blood as a marker of infection in patients with community-acquired pneumonia. J Clin Microbiol 47:3308–3312
10. Rello J, Lisboa T, Lujan M, Gallego M, Kee C, Kay I et al (2009) Severity of pneumococcal pneumonia associated with genomic bacterial load. Chest 136:832–840
11. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R (2008) Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 46:174–182
12. National Committee for Clinical Laboratory Standards (NCCLS) (2008) Performance standards for antimicrobial susceptibility testing: eighteenth informational supplement. CLSI document M100-S18 (ISBN 1-5-56238-653-0). Clinical and Laboratory Standards Institute, Wayne, PA
13. Tarragó D, Fenoll A, Sánchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C et al (2008) Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. Clin Microbiol Infect 14(9):828–834
14. Walker JA, Allen RL, Falmagne P, Johnson MK, Boulnois GJ (1987) Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*. Infect Immun 55:1184–1189
15. Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB (2001) Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 39:1553–1558
16. American Academy of Pediatrics. Committee on Infectious Diseases (2000) Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine (Prevnar), pneumococcal polysaccharide vaccine, and antibiotic prophylaxis. Pediatrics 106:362–366
17. Avni T, Mansur N, Leibovici L, Paul M (2010) PCR using blood for diagnosis of invasive pneumococcal disease: systematic review and meta-analysis. J Clin Microbiol 48:489–496
18. Anh DD, Kilgore PE, Slack MP, Nyambat B, Tho le H, Yoshida LM et al (2009) Surveillance of pneumococcal-associated disease among hospitalized children in Khanh Hoa Province, Vietnam. Clin Infect Dis 48:S57–S64
19. Azzari C, Moriondo M, Indolfi G, Massai C, Becciolini L, de Martino M et al (2008) Molecular detection methods and serotyping performed directly on clinical samples improve diagnostic sensitivity and reveal increased incidence of invasive disease by *Streptococcus pneumoniae* in Italian children. J Med Microbiol 57:1205–1212
20. Carvalho Mda G, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW et al (2007) Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. J Clin Microbiol 45:2460–2466
21. Sheppard CL, Harrison TG, Morris R, Hogan A, George RC (2004) Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. J Med Microbiol 53:189–195
22. Michelow IC, Lozano J, Olsen K, Goto C, Rollins NK, Ghaffar F et al (2002) Diagnosis of *Streptococcus pneumoniae* lower respiratory infection in hospitalized children by culture, polymerase chain reaction, serological testing, and urinary antigen detection. Clin Infect Dis 34:E1–E11
23. Lahti E, Mertsola J, Kontiokari T, Eerola E, Ruuskanen O, Jalava J (2006) Pneumolysin polymerase chain reaction for diagnosis of pneumococcal pneumonia and empyema in children. Eur J Clin Microbiol Infect Dis 25:783–789
24. Tzanakaki G, Tsopanomichalou M, Kesanopoulos K, Matzourani R, Sioumala M, Tabaki A et al (2005) Simultaneous single-tube PCR assay for the detection of *Neisseria meningitidis*, *Haemophilus influenzae* type b and *Streptococcus pneumoniae*. Clin Microbiol Infect 11:386–390
25. Dagan R, Shriker O, Hazan I, Leibovitz E, Greenberg D, Schlaeffer F et al (1998) Prospective study to determine clinical

relevance of detection of pneumococcal DNA in sera of children by PCR. J Clin Microbiol 36:669–673
26. Murdoch DR, Anderson TP, Beynon KA, Chua A, Fleming AM, Laing RT et al (2003) Evaluation of a PCR assay for detection of *Streptococcus pneumoniae* in respiratory and nonrespiratory samples from adults with community-acquired pneumonia. J Clin Microbiol 41:63–66
27. Abdeldaim GM, Strålin K, Olcén P, Blomberg J, Herrmann B (2008) Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. Diagn Microbiol Infect Dis 60:143–150
28. Simões AS, Sá-Leão R, Eleveld MJ, Tavares DA, Carriço JA, Bootsma HJ et al (2010) Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol 48:238–246
29. La Scolea LJ Jr, Dryja D (1984) Quantitation of bacteria in cerebrospinal fluid and blood of children with meningitis and its diagnostic significance. J Clin Microbiol 19:187–190
30. van Haeften R, Palladino S, Kay I, Keil T, Heath C, Waterer GW (2003) A quantitative LightCycler PCR to detect *Streptococcus pneumoniae* in blood and CSF. Diagn Microbiol Infect Dis 47:407–414
31. Kee C, Palladino S, Kay I, Pryce TM, Murray R, Rello J et al (2008) Feasibility of real-time polymerase chain reaction in whole blood to identify *Streptococcus pneumoniae* in patients with community-acquired pneumonia. Diagn Microbiol Infect Dis 61:72–75
32. Abdeldaim G, Herrmann B, Mölling P, Holmberg H, Blomberg J, Olcén P et al (2010) Usefulness of real-time PCR for *lytA*, *ply*, and Spn9802 on plasma samples for the diagnosis of pneumococcal pneumonia. Clin Microbiol Infect 16:1135–1141
33. Wheeler J, Murphy OM, Freeman R, Kearns AM, Steward M, Lee MJ (2000) PCR can add to detection of pneumococcal disease in pneumonic patients receiving antibiotics at admission. J Clin Microbiol 38:3907
34. Carrol ED, Guiver M, Nkhoma S, Mankhambo LA, Marsh J, Balmer P et al (2007) High pneumococcal DNA loads are associated with mortality in Malawian children with invasive pneumococcal disease. Pediatr Infect Dis J 26:416–422
35. Darton T, Guiver M, Naylor S, Jack DL, Kaczmarski EB, Borrow R et al (2009) Severity of meningococcal disease associated with genomic bacterial load. Clin Infect Dis 48(5):587–594
36. Avansino JR, Goldman B, Sawin RS, Flum DR (2005) Primary operative versus nonoperative therapy for pediatric empyema: a meta-analysis. Pediatrics 115:1652–1659

Influence of pneumococcal vaccine on the incidence of empyema

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Purpose of review

The aim of this review is to highlight recent reports (2009) concerning empyema and the heptavalent pneumococcal conjugate vaccine.

Recent findings

Streptococcus pneumoniae remains the most common cause of complicated pneumonia worldwide. Moreover, the incidence of empyema is increasing in many parts of the world and nonvaccine pneumococcal serotypes have been related with this increase. The introduction of heptavalent pneumococcal conjugate vaccine has been associated with the replacement phenomenon in the nasopharynx. Replacement implies that nonvaccine serotypes acquire an ecological advantage for colonizing the nasopharynx and, consequently, increase the carriage status and, in a second step, the disease. Pneumonia with or without empyema has been the main clinical presentation related with the emergence of nonvaccine serotypes. The replacement phenomenon could be multifactorial because other factors apart from heptavalent pneumococcal conjugate vaccine can also contribute to this event.

Summary

A new generation of conjugate vaccines that include new serotypes and a wider spectrum of coverage, and the protein-based vaccines that may prevent invasion and preserve colonization, should help us to achieve a positive long-term impact of pneumococcal vaccination.

Keywords

empyema, pneumococcal serotypes, pneumococcal vaccine

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Introduction

Complicated pneumonia with empyema is a major cause of morbidity and mortality worldwide. Incidence of empyema is increasing in many parts of the world despite the use of the currently available pneumococcal conjugate vaccine, which includes seven serotypes (PCV7). The aim of this review is to highlight recent reports (2009) concerning empyema and pneumococcal vaccination.

Global burden of invasive pneumococcal disease

Streptococcus pneumoniae remains the most common cause of community-acquired pneumonia and complicated pneumonia worldwide. A recent report of the *Haemophilus influenzae* (Hib) and pneumococcal global disease burden working group with staff members of WHO estimated that in the year 2000, there would have been around 11–18 million episodes of serious pneumococcal diseases in children less than 5 years of age [1••]. Since the mid-1980s, the polysaccharide pneumococcal vaccine that contains purified capsular polysaccharides from 23 pneumococcal serotypes has been available. However, polysaccharide vaccine is not efficacious for children less than 2 years of age and does not induce mucosal immunity.

In the year 2000, a protein–polysaccharide conjugate vaccine against the serotypes 4, 6B, 9V, 14, 18C, 19F and 23F (PCV7) was licensed for use in infants and young children. The serotypes included in the PCV7 are most frequently involved in pediatric invasive diseases and detected frequently in the nasopharynx of healthy children. PCV7 has been proved to be well tolerated, immunogenic and effective in preventing pneumococcal disease caused by vaccine serotypes in children [2,3]. In addition, PCV7 also prevents invasive pneumococcal disease (IPD) in adult contacts and nonvaccinated siblings through indirect effect (herd immunity) on pneumococcal transmission [4••]. Despite the effectiveness of the conjugate vaccine, which has allowed a significant decrease in IPD, the emergence of complicated pneumonia is a fact around the world, including countries with implementation of conjugate vaccine [5–9]. Significant differences are observed among countries, and in specific geographical areas the emergence of empyema has become an important health problem. At present,

there are recognized 92 serotypes of pneumococci, including the new serotype 6D, discovered in the last year [10]. Pneumococci serotypes have different capacity to cause invasive and mucosal diseases. PCV7 elicits mucosal immune response and is effective in eradicating vaccine pneumococcal serotypes that are colonizing the human nasopharynx. However, the introduction of PCV7 has been associated with the replacement phenomenon.

Replacement phenomenon

Replacement implies that nonvaccine serotypes acquire an ecological advantage for colonizing the nasopharynx and, consequently, increase the carriage status and, in a second step, the disease. New data about the host–pathogen interaction show evidence of survival strategies of pneumococci and other virulent pathogens for persisting as colonizers in the nasopharynx. Hanage *et al.* [11••] identified a history of hyperrecombination among pneumococcal strains and other related species. The authors suggest that the tolerance of foreign DNA could be an important event for adaptation to environmental pressures such as pneumococcal vaccination and antibiotic treatment. The high diversity of pneumococci implies that heptavalent conjugate vaccine (PCV7) could not reduce the overall prevalence of nasopharyngeal colonization. Serotypes included in PCV7 are replaced by other serotypes and, moreover, by other pathogens [12•,13•,14]. The clinical consequences depend on the capacity to produce disease of nonvaccine serotypes. Replacement by a nonvirulent pathogen would be good news, but replacement by a potential pulmonary or extrapulmonary pathogen would be bad news and imply the reemergence of invasive disease. In addition, changes in the main nasopharyngeal colonizers may be associated with changes in clinical and epidemiological factors. A recent study that compared disease caused by invasive serogroups 1, 5 and 7 (not included in vaccine) and the other serogroups (including PCV7 serotypes) [15•] found that invasive serogroups caused pneumonia more often than other serogroups. Therefore, the replacement by these invasive serotypes is associated, at least in part, with the dramatic increase of empyema in different geographical areas. Recent reports from Barcelona and Utah confirm the increase of pneumonia and complicated pneumonia (with empyema) related with the emergence of serotype 1 circulation after introduction of PCV7 [16,17].

Increase of multiresistant clones expressing nonvaccine serotypes

The increase of multiresistant clones expressing nonvaccine serotypes is of special concern. The development of antibiotic resistance is another survival strategy of pneumococci. Serotype 1 has a low fatality rate and is rarely associated with penicillin resistance. However, other nonvaccine serotypes such as 19A, 15A, 6A/C or 35B harboring reduced antibiotic susceptibility have expanded in the vaccine era. This expansion has been observed in nasopharyngeal carriers [12•,13•,18] and also in IPD [19,20,21••,22,23]. The selection of penicillin nonsusceptibility strains may be explained by the high rates of antibiotic used mainly in children with otitis media or upper respiratory tract infection. In the prevaccine era, some multiresistant clones had been reported worldwide. The Pneumococcal Molecular Epidemiology Network (PMEN) has recognized more than 26 pneumococcal multiresistant clones disseminated in two or more continents [24]. The world spread of multiresistant clones implies that pneumococcal resistance may also be a problem in countries with a low antibiotic use. In the prevaccine period, the majority of resistant clones were expressing serotypes included in PCV7 such as serotype 6B, 9V, 19F and 23F [25,26]. In the vaccine period, several authors have reported that some international multiresistant clones or closely related strains are expanding but mainly expressing nonvaccine serotypes [7,16,19,20,22,27,28]. These strains shared the antibiotic resistant strategy and the expression of a serotype not covered by the vaccine, which could enable world dissemination, including countries without vaccine use [29]. Furthermore, the capacity of producing serious invasive disease such as meningitis, sepsis or empyema is reported elsewhere. Up to date, the main multiresistant emergent nonvaccine serotype is serotype 19A. The majority of these emergent strains show the expansion of preexistent clones, but some reports have also shown a capsular switching mechanism that is contributing to the increase in serotype 19A [30]. Capsular switch is a recombinant event that could imply that DNA fragment containing the capsular locus is transferred between strains. The result is that the molecular study of some 19A isolates shows clones or sequence type that were only originally identified in vaccine serotype strains in the prevaccine era. Although serotype 19A has already been shown to be the main multiresistant emergent nonvaccine serotype in the vaccine era, other serotypes could have similar invasive potential. More striking are the data of a recent study published by Simões *et al.* [31•]. The authors report a significant increase in highly penicillin-resistant and multidrug-resistant pneumococcal-like strains in healthy nasopharyngeal carrier children in Oeiras, Portugal. The authors have been performing colonizing surveillance studies since 1996, and only in 2006 identified these closely related pneumococcal strains. They hypothesize that these changes in the nasopharynx ecosystem might be related to the introduction of PCV7, which could favor the colonization of these pneumococcal-like strains. However, it remains to be ascertained whether or not these quasi-pneumococcal strains may cause disease. Continued surveillance of changes in the ecological niche of the nasopharynx and identification of the clinical role

of these pathogens are mandatory for the forthcoming years.

Replacement: a multifactorial event

There has been an association between the introduction of PCV7 in 2001 and the expansion of nonvaccine serotypes and the evidence of increased genetic diversity in different clonal types expressing nonvaccine serotypes. However, multiple factors could be related with this event.

A high disparity has been reported in the magnitude of replacement around the world, including closed geographical areas. The increase in empyema rates and, moreover, the increase in deaths caused by empyema is a reality in Utah and California [6,17,32••,33•]. However, a recent study performed in other eight counties of the United States has shown a significant decrease in invasive pneumococcal pneumonia (and overall IPD), comparing rates of disease in 1998–1999 with those in 2007 [2]. If conjugate vaccine were exclusively related with replacement, the emergence of noninvasive serotypes would be more universal, especially in populations with wide use of the vaccine.

Natural selection of any live organism is extremely complex. Multiple factors are involved in the selection of the best adapted organisms in a geographical area or in a specific period of time. The environmental conditions of the nasopharyngeal ecological niche are changing over time. It has been recognized that different risk factors contribute to a high rate of nasopharyngeal carriers and to the development of pneumonia. Underlying chronic diseases, genetic factors, ethnicity, malnutrition, low birth weight, lack of breastfeeding, lack of measles immunization, indoor air pollution, antibiotic use or day care attendance have all been identified to be associated with developing IPD [34–41]. A recent report from Alaska [42••] has found an increase in IPD rates in the vaccine period associated with lack of in-home piped water. Lack of running water is also associated with limitations of handwashing and low per capita income. On the other hand, an increase in pneumococcal pneumonia has also been reported in Olmsted county, Minnesota [43••], a county with high educational levels. Data from Alaska and Minnesota emphasize the multiple and probably unknown local factors that could be associated with the emergence of an infectious disease such as pneumococcal pneumonia. If specific local factors are associated with the natural selection of local prevalent pathogens, epidemiological surveillance and molecular surveillance will be necessary in different geographical areas, mainly in developing countries with high burden of IPDs [1••,44]. In addition, a broader standardization of case definition and data reporting of IPD is also needed for understanding the temporal and geographical trends of different clonal types and serotypes of pneumococci.

Recent reports have suggested that the use of pneumococcal vaccine may be associated with changes in clinical management of the patients. For example, well appearing young children who have received PCV7 and are attended because of fever without source could have blood cultures performed less frequently, and could be sent home without antimicrobial therapy more frequently, than nonvaccinated children with similar clinical conditions [45]. This strategy may be appropriate in areas in which pneumococcal vaccine impact is high, but could not be applied in areas in which emergence of pneumococcal infection caused by nonvaccine serotypes is becoming a problem.

Future of vaccine strategies

IPD is a preventable disease. Huge benefits have been achieved due to vaccination against infectious diseases, including pneumococcal disease. The extremely high burden of IPD, mainly pneumococcal pneumonia, and the number of annual deaths by this disease imply that pneumococcal disease prevention must be accelerated. Overall, the benefits of PCV7 have been undoubted in a significant number of children, but the versatility and capacity of pneumococci to escape from vaccine action require a new generation of vaccines urgently.

The serotype coverage of PCV10 (which included the seven serotypes of PCV7 and serotypes 1, 5 and 7F) and specially PCV13 (PCV10 and additional serotypes 3, 6A and 19A) has been expected to provide a major protection against pneumococcal infection, due to the major number of serotypes covered by these vaccines. Depending on the region, the serotype coverage of these vaccines could be around 60–80% [46]. However, surveillance epidemiological studies are required to evaluate the potential impact of the new generation of conjugate pneumococcal vaccines on the replacement phenomenon.

S. pneumoniae has two main types of virulence factors: the high heterogeneous capsule that is the target of the current conjugate vaccines, and the protein-surface or subsurface proteins, such as pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumolysin (Ply), pneumococcal adherence and virulence factor A (PavA), pneumococcal choline binding protein A (PcpA), choline binding protein A (CbpA/PSPC) and pneumococcal serine-rich repeat protein (PsrP), among others [47–50,51•]. Specifically, some of these proteins play vital roles in the adherence of the pneumococcus and internalization in epithelial cells of the nasopharynx. This fact is an important step toward systemic spread. Recently, some of these proteins have

been postulated as vaccine candidates. The possibility of a vaccine only against pneumococci determinants involved in the process of invasion (for example, avoiding adherence of pneumococci to lung cells) but without disturbing colonizers of the ecological niche of the nasopharynx opens a potential solution for combating the replacement event. It is probable that these protein vaccines could help to reduce the burden of pneumococcal disease in the near future.

Conclusion

PCV7 is highly effective against IPD produced by serotypes included in the vaccine. However, the high genetic diversity of *S. pneumoniae* and the complex host–pathogen interaction related with the production of invasive disease lead to important differences observed with regard to the impact of PCV7 in different populations. The increase in IPD and empyema due to non-PCV7 is an important problem and should be taken into consideration. A new generation of conjugate vaccines that include new serotypes and a wider spectrum of coverage, and the protein-based vaccines that may prevent invasion and preserve colonization, should help us to achieve a positive long-term impact of pneumococcal vaccination.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 413).

1 O'Brien KL, Wolfson LJ, Watt JP, *et al.* Burden of disease caused by •• *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009; 374:893–902.
The authors estimated the burden of pneumococcal disease and deaths in children less than 5 years of age for the year 2000 at global and country levels by systematic methods including a literature review. The conclusion of this study is that *S. pneumoniae* causes around 11% (8–12%) of all deaths in children aged 1–59 months.

2 Pilishvili T, Lexau C, Farley MM, *et al.* Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010; 201:32–41.

3 Lucero MG, Dulalia VE, Nillos LT. Pneumococcal conjugate vaccines for preventing vaccine-type invasive pneumococcal disease and X-ray defined pneumonia in children less than two years of age. Cochrane Database Syst Rev 2009:CD004977.

4 Pulido M, Sorvillo F. Declining invasive pneumococcal disease mortality in the •• United States, 1990–2005. Vaccine 2010; 28:889–892.
The authors examined PCV7 influence on IPD mortality for all age groups using a National Multiple-Cause-of-Death data (1990–2005). Age-specific mortality rates were compared before and after PCV7 use. The study reports a decrease in age-specific mortality rates after the introduction of PCV7.

5 Eastham KM, Freeman R, Kearns AM, *et al.* Clinical features, aetiology and outcome of empyema in children in the north east of England. Thorax 2004; 59:522–525.

6 Byington CL, Samore M, Stoddard GJ, *et al.* Temporal trends of invasive disease due to Streptococcus pneumoniae among children in the intermountain west: emergence of nonvaccine serogroups. Clin Infect Dis 2005; 41:21–29.

7 Muñoz-Almagro C, Jordan I, Gene A, *et al.* Emergence of invasive pneumococcal disease caused by nonvaccine serotypes in the era of 7-valent conjugate vaccine. Clin Infect Dis 2008; 46:174–182.

8 Ardanuy C, Tubau F, Pallares R, *et al.* Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997–2007. Clin Infect Dis 2009; 48:57–64.

9 Lin CJ, Chen PY, Huang FL, *et al.* Radiographic, clinical, and prognostic features of complicated and uncomplicated community-acquired lobar pneumonia in children. J Microbiol Immunol Infect 2006; 39:489–495.

10 Jin P, Kong F, Xiao M, *et al.* First report of putative Streptococcus pneumoniae serotype 6D among nasopharyngeal isolates from Fijian children. J Infect Dis 2009; 200:1375–1380.

11 Hanage WP, Fraser C, Tang J, *et al.* Hyper-recombination, diversity, and •• antibiotic resistance in pneumococcus. Science 2009; 324:1454–1457.
The authors analyzed 1930 pneumococcal genotypes and 94 genotypes from related species and identified mosaic genotypes representing admixture between populations. The authors hypothesize that pneumococcal and related species are more likely to acquire both divergent genetic material and resistance determinants. This finding could have consequences for the reemergence of drug resistance after pneumococcal vaccination.

12 Sá-Leão R, Nunes S, Brito-Avô A, *et al.* Changes in pneumococcal serotypes • and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. Clin Microbiol Infect 2009; 15:1002–1007.
The authors report pneumococcal carriage rates, serotypes and antibiotypes determined in 2001 and 2006. The pneumococci carriage rate remained stable. A serotype replacement was observed among vaccinated and unvaccinated children.

13 Huang SS, Hinrichsen VL, Stevenson AE, *et al.* Continued impact of pneu- • mococcal conjugate vaccine on carriage in young children. Pediatrics 2009; 124:e1–e11.
A low prevalence (3%) of vaccine serotypes has been found in nasopharyngeal samples of children in 2006–2007. A rapid replacement with penicillin-nonsusceptible nonvaccine serotypes, particularly 19A and 35B, has occurred.

14 Murphy TF, Bakaletz LO, Smeesters PR. Microbial interactions in the respiratory tract. Pediatr Infect Dis J 2009; 28:S121–S126.

15 Trollfors B, Berg S, Backhaus E, *et al.* Invasive, paediatric, vaccine strains of • Streptococcus pneumoniae: are there differences in clinical characteristics? Scand J Infect Dis 2009; 41:84–87.
The authors reported that infections caused by invasive pneumococcal serogroups 1 and 7 caused pneumonia more often than other serogroups.

16 Hernandez-Bou S, Garcia-Garcia JJ, Esteva C, *et al.* Pediatric parapneumonic pleural effusion: epidemiology, clinical characteristics, and microbiological diagnosis. Pediatr Pulmonol 2009; 44:1192–1200.

17 Byington CL, Hulten KG, Ampofo K, *et al.* Molecular epidemiology of pediatric pneumococcal empyema 2001-2007. J Clin Microbiol 2010; 48:520–525.

18 Leach AJ, Morris PS, McCallum GB, *et al.* Emerging pneumococcal carriage serotypes in a high-risk population receiving universal 7-valent pneumococcal conjugate vaccine and 23-valent polysaccharide vaccine since 2001. BMC Infect Dis 2009; 9:121.

19 Muñoz-Almagro C, Esteva C, de Sevilla MF, *et al.* Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. J Infect 2009; 59:75–82.

20 Song JH, Baek JY, Cheong HS, *et al.* Changes of serotype and genotype in Streptococcus pneumoniae isolates from a Korean hospital in 2007. Diagn Microbiol Infect Dis 2009; 63:271–278.

21 Richter SS, Heilmann KP, Dohrn CL, *et al.* Changing epidemiology of anti- •• microbial-resistant Streptococcus pneumoniae in the United States, 2004–2005. Clin Infect Dis 2009; 48:e23–e33.
The authors found that serotype distribution of the penicillin-resistant *S. pneumoniae* population changed from 1999–2000 to 2004–2005, with an increase in the prevalence of serotype 19A (1.5–35.4%) and serotype 35B (1.2–12.5%). The authors also found a decrease in the prevalence of most penicillin-resistant PCV7 serotypes.

22 Mahjoub-Messai F, Doit C, Koeck JL, *et al.* Population snapshot of Streptococcus pneumoniae serotype 19A isolates before and after introduction of seven-valent pneumococcal vaccination for French children. J Clin Microbiol 2009; 47:837–840.

23 Techasaensiri C, Messina AF, Katz K, *et al.* Epidemiology and evolution of invasive pneumococcal disease caused by multidrug resistant serotypes of 19A in the 8 years after implementation of pneumococcal conjugate vaccine immunization in Dallas, Texas. Pediatr Infect Dis J 2009 [Epub ahead of print].

24 McGee L, McDougal L, Zhou J, *et al.* Nomenclature of major antimicrobial-resistant clones of *Streptococcus pneumoniae* defined by the Pneumococcal Molecular Epidemiology Network. J Clin Microbiol 2001; 39:2565–2571.

25 Isaacman DJ, McIntosh ED, Reinert RR. Burden of invasive pneumococcal disease and serotype distribution among *Streptococcus pneumoniae* isolates in young children in Europe: impact of the 7-valent pneumococcal conjugate vaccine and considerations for future conjugate vaccines. Int J Infect Dis 2009 [Epub ahead of print].

26 Richter SS, Heilmann KP, Coffman SL. The molecular epidemiology of penicillin-resistant *Streptococcus pneumoniae* in the United States. Clin Infect Dis 2002; 34:330–339.

27 Aguiar SI, Pinto FR, Nunes S, *et al.* Increase of Denmark14-230 clone as a cause of pneumococcal infection in Portugal within a background of diverse serotype 19A lineages. J Clin Microbiol 2010; 48:101–108.

28 Ardanuy C, Rolo D, Fenoll A. Emergence of a multidrug-resistant clone (ST320) among invasive serotype 19A pneumococci in Spain. J Antimicrob Chemother 2009; 64:507–510.

29 Dagan R, Givon-Lavi N, Leibovitz E. Introduction and proliferation of multidrug-resistant *Streptococcus pneumoniae* serotype 19A clones that cause acute otitis media in an unvaccinated population. J Infect Dis 2009; 199:776–785.

30 Brueggemann AB, Pai R, Crook DW, *et al.* Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. PLoS Pathog 2007; 3:e168.

31 • Simões AS, Sá-Leão R, Eleveld MJ, *et al.* Highly penicillin-resistant multidrug-resistant pneumococcus-like strains colonizing children in Oeiras, Portugal: genomic characteristics and implications for surveillance. J Clin Microbiol 2010; 48:238–246.
The authors found an increase from 0.7% in 2003 to 5% in 2006 in the prevalence of penicillin resistance of pneumococcus-like strains classified by phenotypic and genotypic methods as 'streptococci of the mitis group'.

32 •• Bender JM, Ampofo K, Sheng X, *et al.* Parapneumonic empyema deaths during past century, Utah. Emerg Infect Dis 2009; 15:44–48.
The authors analyzed the historical deaths caused by empyema in the past 100 years from Utah, United States. Deaths caused by empyema have increased in 2000–2004 when compared with the historic rates. In addition, the authors analyzed the historical relationship between deaths due to empyema and influenza pandemics in the same period.

33 • Li ST, Tancredi DJ. Empyema hospitalizations increased in US children despite pneumococcal conjugate vaccine. Pediatrics 2010; 125:26–33.
The authors found an increase of 70% in the annual empyema hospitalization rates between 1997 and 2006 in children less than 18 years of age.

34 Burman LA, Norrby R, Trollfors B. Invasive pneumococcal infections incidence, predisposing factors, and prognosis. Rev Infect Dis 1985; 7:133–142.

35 Davidson M, Parkinson A, Bulkow LR, *et al.* The epidemiology of invasive pneumococcal disease in Alaska, 1986–1990: ethnic differences and opportunities for prevention. J Infect Dis 1994; 170:368–376.

36 Von Bermuth H, Picard C, Zhongbo J, *et al.* Pyogenic bacterial infections in humans with MyD88 deficiency. Science 2008; 321:691–696.

37 Levine OS, Farley M, Harrison LH, *et al.* Risk factors for invasive pneumococcal disease in children: a population-based case–control study in North America. Pediatrics 1999; 103:E28.

38 Hjuler T, Wohlfahrt J, Simonsen J, *et al.* Perinatal and crowding-related risk factors for invasive pneumococcal disease in infants and young children: a population-based case–control study. Clin Infect Dis 2007; 44:1051–1056.

39 Hjuler T, Wohlfahrt J, Kaltoft MS, *et al.* Risks of invasive pneumococcal disease in children with underlying chronic diseases. Pediatrics 2008; 122:E26–E32.

40 Nohynek H, Madhi S, Grijalva CG. Childhood bacterial respiratory diseases: past, present, and future. Pediatr Infect Dis J 2009; 28 (10 Suppl):S127–S132.

41 Samore MH, Lipsitch M, Alder S, *et al.* Mechanisms by which antibiotics promote dissemination of resistant pneumococci in human populations. Am J Epidemiol 2006; 163:160–170.

42 •• Wenger JD, Zulz T, Bruden D, *et al.* Invasive pneumococcal disease in Alaskan children: impact of the seven-valent pneumococcal conjugate vaccine and the role of water supply. Pediatr Infect Dis J 2010; 29:251–256.
This study evaluates the association of IPD rates and serotype distribution with immunization, socioeconomic status and in-home water service in Alaskan children less than 5 years of age. The conclusions were that high IPD rates in Alaska were associated with lack of in-home piped water. Lack of running water is also associated with limitations of handwashing and low per capita income.

43 •• Tsigrelis C, Tleyjeh IM, Lahr BD, *et al.* Trends in invasive pneumococcal disease among older adults in Olmsted County, Minnesota. J Infect 2009; 59:188–193.
This study identifies IPD in adults aged more or equal to 50 years. The authors report a 45% increase in the incidence rate of IPD from 2001–2003 (17.7 cases per 100 000 person-years) to 2004–2007 (32.1 cases per 100 000 person-years). The increase in IPD is due to nonvaccine PCV7 serotypes.

44 Naheed A, Saha SK, Breiman RF, *et al.* Multihospital surveillance of pneumonia burden among children aged <5 years hospitalized for pneumonia in Bangladesh. Clin Infect Dis 2009; 48 (Suppl 2):S82–S89.

45 Chiappini E, Galli L, Bonsignori F, *et al.* Self-reported pediatricians' management of the well appearing young child with fever without a source: first survey in an European country in the antipneumococcal vaccine era. BMC Public Health 2009; 9:300.

46 PneumoADIP. Pneumococcal diseases. http://www.preventpneumo.org/diseases/pneumococcal_diseases/index.cfm [Accessed 22 December 2009].

47 Kadioglu A, Weiser JN, Paton JC, Andrew PW. The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nature Rev Microbiol 2008; 6:288–301.

48 Rajam G, Anderton JM, Carlone GM, *et al.* Pneumococcal surface adhesin A (PsaA): a review. Crit Rev Microbiol 2008; 34 (3–4):131–142.

49 Glover DT, Hollingshead SK, Briles DE. *Streptococcus pneumoniae* surface protein PcpA elicits protection against lung infection and fatal sepsis. Infect Inmun 2008; 76:2767–2776.

50 Shivshankar P, Sanchez C, Rose LF, Orihuela CJ. The *Streptococcus pneumoniae* adhesin PsrP binds to Keratin 10 on lung cells. Mol Microbiol 2009; 73:663–679.

51 • Van der Poll T, Opal SM. Pathogenesis, treatment, and prevention of pneumococcal pneumonia. Lancet 2009; 374:1543–1556.
A good review about the high genetic diversity of pneumococci and the multiple virulence factors of this bacterium.

Canvis en l'epidemiologia del pneumococ en el nostre medi

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Resum

Introducció. Tot i la utilització de la vacuna pneumocòccica conjugada heptavalent (PCV7), l'*Streptococcus pneumoniae* continua sent un important problema de salut pública.

Objectiu. Analitzar l'evolució de la malaltia invasiva pneumocòccica en l'era de la vacuna conjugada heptavalent amb una referència especial a la nostra àrea geogràfica.

Mètode. Revisió no sistemàtica.

Resultats. La introducció de la PCV7 s'ha associat amb el fenomen del reemplaçament de serotips a la nasofaringe. El reemplaçament implica que els serotips no vacunals adquireixen un avantatge ecològic a l'hora de colonitzar la nasofaringe. Com a conseqüència, augmenta la seva prevalença com a portadors i en una segona etapa com a productors de malaltia. La pneumònia, amb empiema o sense, ha estat la principal manifestació clínica relacionada amb la malaltia produïda per serotips no vacunals. El reemplaçament de serotips és un fenomen multifactorial i altres factors diferents a la vacunació també es relacionen amb aquest fet.

Conclusions. Una nova generació de vacunes conjugades que incloguin un rang més ampli de serotips milloraria la cobertura vacunal. D'altra banda, el desenvolupament de vacunes proteiques que frenin la malaltia invasiva, però que preservin la colonització, podria tenir un impacte positiu de la vacunació antipneumocòccica a llarg termini.

Paraules clau: Streptococcus pneumoniae. *Reemplaçament. Serotips. Vacuna. Epidemiologia.*

Cambios en la epidemiología de neumococo en nuestro medio

Introducción. A pesar de la utilización de la vacuna neumocócica conjugada heptavalente (PCV7), *Streptococcus pneumoniae* sigue siendo un importante problema de salud pública.

Objetivo. Analizar la evolución de la enfermedad invasiva neumocócica en la era de la vacuna conjugada heptavalente con especial referencia a nuestra área geográfica.

Método. Revisión no sistemática.

Resultados. La introducción de la PCV7 se ha asociado con el fenómeno de reemplazo de serotipos en nasofaringe. El reemplazo implica que los serotipos no vacunales adquieren una ventaja ecológica para colonizar la nasofaringe. En consecuencia, aumenta su prevalencia como portadores y en una segunda etapa como productores de enfermedad. La neumonía con o sin empiema ha sido la presentación clínica principalmente relacionada con la enfermedad producida por serotipos no vacunales. El reemplazo de serotipos es un fenómeno multifactorial y otros factores distintos a la vacunación están también relacionados con este suceso.

Conclusiones. Una nueva generación de vacunas conjugadas que incluyan un mayor rango de serotipos mejoraría la cobertura vacunal. Por otro lado, el desarrollo de vacunas proteicas que impidan la enfermedad invasiva pero que preserven la colonización, podría tener un impacto positivo de la vacunación antineumocócica a largo plazo.

Palabras clave: Streptococcus pneumoniae. *Reemplazo. Serotipos. Vacuna. Epidemiología.*

Changes in pneumococcal epidemiology in our geographical area

Introduction. Despite the use of pneumococcal conjugate vaccine (PCV7) *Streptococcus pneumoniae* remains a major public health problem.

Objective. To highlight recent reports concerning invasive pneumococcal disease in the era of heptavalent conjugate vaccine in our geographical area.

Method. Non-systematic review.

Results. The introduction of PCV7 has been associated with the replacement phenomenon in nasopharynx. Replacement implies that non-vaccine serotypes acquire an ecological advantage for colonizing the nasopharynx and, consequently, increase their carriage prevalence and in a second step, the disease. Pneumonia with or without empyema has been the main clinical presentation related with the emergence of non-vaccine serotypes. Replacement is a multifactorial event and other factors unrelated to PCV7 are also responsible for this effect.

Conclusions. A new generation of conjugate vaccines, which includes new serotypes and a wider spectrum of coverage, as well as the protein-based vaccines that may prevent invasion and preserve colonization, should help us achieve a positive long-term impact of pneumococcal vaccination.

Key words: Streptococcus pneumoniae. *Replacement. Serotypes. Vaccine. Epidemiology.*

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Introducció

La infecció per *Streptococcus pneumoniae* o pneumococ és un greu problema de salut pública. Un informe recent elaborat per membres de l'Organització Mundial de la Salut ha estimat que cada any es produeixen entre 11 i 18 milions d'episodis greus de malaltia pneumocòccica en el món [1].

El 2 de febrer de 2001 la Comissió Europea va autoritzar la comercialització de la vacuna pneumocòccica conjugada heptavalent (PCV7), que incloïa set serotips seleccionats pel fet de ser els que provocaven més sovint la malaltia greu en infants (serotips 4, 6B, 9V, 14, 18C, 19F i 23F). Deu anys després de la seva comercialització, aquesta vacuna ha mostrat ser segura, immunogènica i eficaç en la prevenció de la malaltia pneumocòccica produïda pels serotips inclosos a la vacuna [2]. També s'ha observat una reducció de la malaltia per serotips vacunals en adults, per un efecte d'immunitat de grup, en reduir la transmissió dels set serotips a la comunitat [3]. Tot i l'eficàcia de la vacuna conjugada, que ha permès una disminució significativa de la malaltia pneumocòccica invasiva, diferents autors han observat un increment de la malaltia invasiva pneumocòccica com a conseqüència de l'augment de serotips no inclosos a la vacuna. L'augment de la malaltia s'ha observat a Catalunya, tant en nens com en adults, així com en altres àrees geogràfiques [4-10]. Aquest augment està relacionat, en part, amb l'elevada diversitat del genoma del pneumococ. Actualment es coneixen més de 90 serotips de *S. pneumoniae* amb una capacitat diferent de produir malaltia invasiva. Una conseqüència inesperada de la introducció de la PCV7 és el fenomen del reemplaçament de serotips.

Reemplaçament de serotips

El reemplaçament de serotips té lloc perquè la vacuna conjugada heptavalent també és eficaç en l'eradicació de soques de pneumococ colonitzants a la nasofaringe, que expressen serotips inclosos a la vacuna. Si desapareixen els serotips vacunals del nínxol ecològic de la nasofaringe humana, la resta de serotips, adquireixen un avantatge ecològic. Com a conseqüència, la seva prevalença com a colonitzants augmenta, de manera que poden causar malaltia en una segona etapa. L'efecte d'aquest fenomen és que la PCV7 no redueix la prevalença global de la colonització a la nasofaringe, sinó que uns serotips són substituïts per uns altres serotips o bé, fins i tot, per uns altres patògens [11-12]. El reemplaçament a la nasofaringe per serotips no virulents seria una bona notícia, però la substitució d'aquests per serotips amb capacitat de produir malaltia implica l'anul·lació a llarg termini de l'efecte de la vacuna. D'altra banda, es coneix que no tots els serogrups o serotips produeixen el mateix tipus de malaltia invasiva. Els serogrups no vacunals 1, 5 i 7, que són considerats serogrups virulents amb una capacitat invasiva elevada, s'associen amb freqüència a malaltia pulmonar [13], mentre que s'ha observat que els serotips vacunals produeixen majoritàriament bacterièmia oculta en infants menors de dos anys. El recanvi de serotips està associat, per tant, a canvis en les manifestacions clíniques i a canvis epidemiològics. A la nostra àrea geogràfica hem observat aquest augment de la malaltia per serotips no vacunals, que a més a més s'ha vist relacionat amb un canvi en la distribució de les principals manifestacions clíniques de la malaltia invasiva i en el grup d'edat de més risc de malaltia. En l'era prevacunal, les taxes més altes d'incidència de la malaltia s'observaven en infants menors de dos anys, i la bacterièmia oculta era la manifestació clínica principal. A l'era de la vacuna conjugada, l'augment de la incidència de la malaltia invasiva s'ha observat principalment en infants de més de dos anys, i la principal manifestació clínica és la pneumònia. Aquest fet és degut parcialment a l'emergència del serotip 1 i en concret a la introducció l'any 2000 d'un clon virulent (ST306) amb una capacitat invasiva elevada [4, 14].

El reemplaçament de serotips que es va observar a Catalunya, en un primer moment va ser causat fonamentalment per clons virulents que expressaven els serotips epidèmics 1 i 5, aquest últim sobretot en la població adulta. Els últims anys s'ha detectat també l'augment d'altres serotips no vacunals com el serotip 19A [15]. L'emergència del serotip 19A és deguda en gran part a l'expansió de clons amb resistència múltiple, que a l'era prevacunal expressaven serotips vacunals i que ara són detectats majoritàriament expressant aquest serotip 19A, o altres de no vacunals. El fenomen d'expansió de soques no vacunals amb resistència múltiple s'ha observat a diferents àrees geogràfiques, tant en portadors nasofaringis [11-12, 16], com en pacients amb malaltia invasiva [15, 17-20]. Fins avui, el principal serotip emergent productor de malaltia invasiva, amb aquestes característiques de multiresistència, és el serotip 19A.

La disseminació de clons multiresistents preocupa especialment, ja que el desenvolupament de resistència a antibiòtics és una estratègia de supervivència del pneumococ que complica la teràpia antimicrobiana dels malalts. Els serotips epidèmics 1 i 5 s'associen a una baixa taxa de mortalitat i no acostumen a ser colonitzants en els infants petits (la principal via de transmissió de la malaltia). A més a més, aquests dos serotips són majoritàriament sensibles a penicil·lina. La baixa prevalença com a colonitzants nasofaringis explica que aquests serotips causin brots locals, però que la seva disseminació a gran escala, en principi, sigui més difícil. Tanmateix, un clon amb resistència múltiple que expressi serotips fàcilment detectats en portadors, com els 19A, 15A, 6A/C i 35B, pot esdevenir un problema de disseminació més global. Estudis fets a l'era prevacunal mostren que la propagació mundial de clons multiresistents va generar problemes a països amb un consum baix d'antibiòtics. Per tant, cal esperar, i així ho expliquen alguns autors, que s'observi

una disseminació global amb augment d'aquestes soques no vacunals també a països en què la vacuna no s'ha introduït [21].

El reemplaçament de serotips, un fenomen multifactorial

La coincidència de la introducció de PCV7 el 2001 amb l'emergència de malaltia causada per soques no vacunals, així com la gran diversitat genètica de *S. pneumoniae* que s'ha observat els últims anys, són dades que suggereixen que la vacuna conjugada heptavalent ha esdevingut un dels factors relacionat amb el reemplaçament de serotips. Hi ha, però, altres factors que també poden estar relacionats amb l'augment de la malaltia invasiva pneumocòccica per soques no vacunals. Si la vacuna fos l'únic agent causal d'aquesta emergència, les dades dels països amb una implantació similar de la vacuna serien més homogènies. Tanmateix, observem resultats discrepants en àrees geogràfiques properes. Per exemple, a Utah o a Califòrnia l'augment de la pneumònia pneumocòccica complicada és un problema important [7, 22-23], però a altres estats dels Estats Units s'ha confirmat una disminució significativa de la pneumònia pneumocòccica invasiva (i, en general, de la malaltia pneumocòccica invasiva) [2].

La selecció natural de qualsevol organisme viu és un fenomen molt complex. Hi ha múltiples factors que intervenen en la selecció dels organismes més ben adaptats a una àrea geogràfica o a un període determinat. La vacuna conjugada esdevé un nou factor implicat que ha interaccionat amb altres factors com les condicions ambientals de la nasofaringe (per exemple, la interacció amb altres patògens), factors epidemiològics que faciliten la transmissió (una densitat de població elevada facilitaria la disseminació de soques epidèmiques o el consum d'antibiòtics facilitaria el desenvolupament de resistències) o altres factors locals que incideixen en la interacció hoste-patogen. S'ha de considerar l'impacte diferent que ha tingut la vacuna conjugada en una àrea geogràfica concreta a l'hora de prendre decisions clíniques. Estudis recents mostren que l'antecedent de vacunació pot influir en aquestes decisions clíniques. Per exemple, un estudi italià mostra que un infant amb bon aspecte, de menys de dos anys, amb febre sense focus i vacunat, seria donat d'alta a domicili més sovint, sense obtenir mostra d'hemocultiu i sense iniciar tractament antibiòtic, que un pacient amb les mateixes característiques que no hagués estat vacunat [24]. Aquesta estratègia pot ser errònia en el nostre país, on s'ha observat un augment de la malaltia invasiva produïda per serotips no vacunals.

El futur de les estratègies de vacunació

La malaltia pneumocòccica invasiva és una malaltia prevenible. La vacunació és una de les principals accions en la lluita contra les malalties infeccioses. Globalment, la vacuna conjugada heptavalent ha beneficiat un gran nombre d'infants, però la versatilitat i la capacitat dels pneumococs per escapar a l'acció d'aquesta vacuna implica que es necessiti urgentment una nova generació de vacunes, especialment als països amb una càrrega elevada de la malaltia. La cobertura de serotips de les noves vacunes conjugades 10-valent (que inclouen els set serotips de la vacuna PCV7 més els serotips 1, 5 i 7F), i especialment la vacuna 13-valent (que afegeix als de la vacuna 10-valent els serotips 3, 6A i 19A) obren noves esperances per obtenir més protecció. Segons la regió, la cobertura de serotips d'aquestes vacunes s'estima al voltant d'un 60-80% [25]. Tanmateix, fins i tot amb aquesta cobertura més àmplia de serotips, no es pot garantir que el reemplaçament de serotips no pugui reduir l'impacte positiu de les noves vacunes.

S. pneumoniae té dos tipus principals de factors de virulència: la càpsula i les proteïnes de superfície. La càpsula és el component del pneumococ que ens permet classificar-lo en serotips. És el principal factor de virulència i l'objectiu de les actuals vacunes conjugades. L' altre factor de virulència són les proteïnes de superfície o subproteïnes de superfície, per exemple pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), pneumolysin (Ply), pneumococcal adherence and virulence factor A (PavA), pneumococcal choline binding protein A (PcpA) o pneumococcal serine-rich repeat protein (PsrP). Algunes d'aquestes proteïnes estan implicades en l'adherència del pneumococ i en la seva internalització en les cèl·lules epitelials de la nasofaringe, pas fonamental per a la propagació sistèmica. Recentment, algunes d'aquestes proteïnes s'han postulat com a candidates per al desenvolupament d'una vacuna proteica. La possibilitat d'una única vacuna contra determinants del pneumococ que només participen en l'etapa de la invasió (per exemple, evitant l'adherència dels pneumococs a les cèl·lules del pulmó), sense pertorbar els colonitzadors del nínxol ecològic de la nasofaringe, obre una possible solució al fenomen del reemplaçament. Probablement, en un futur, les vacunes proteiques ajudaran a disminuir la càrrega de la malaltia pneumocòccica.

Conclusió

La vacuna conjugada heptavalent és altament eficaç en la prevenció de la malaltia produïda per serotips inclosos a la vacuna. Tanmateix, la gran diversitat genètica del pneumococ i la complexa interacció hoste-patogen fan que l'impacte de la vacuna pugui ser diferent en diferents poblacions. A Catalunya, l'augment de malaltia invasiva per serotips no vacunals és un problema que s'ha de prendre en consideració i que obliga a mantenir una estreta vigilància epidemiològica molecular d'aquest patogen.

Bibliografia

1. O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. Lancet 2009; 374: 893-902.
2. Pilishvili T, Lexau C, Farley MM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010; 201(1): 32-41.
3. Pulido M, Sorvillo F. Declining invasive pneumococcal disease mortality in the United States, 1990-2005. Vaccine 2009; 29 d'octubre [publicació en línia abans de la impressió].
4. Muñoz-Almagro C, Jordan I, Gene A, et al. Emergence of Invasive Pneumococcal Disease Caused by Non-vaccine Serotypes in the Era of 7-Valent Conjugate Vaccine. Clin Infect Dis 2008; 46: 174-182.
5. Ardanuy C, Tubau F, Pallares R. Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997-2007. Clin Infect Dis 2009; 48(1): 57-64.
6. Eastham KM, Freeman R, Kearns AM, et al. Clinical features, etiology and outcome of empyema in children in the north east of England. Thorax 2004; 59: 522-525.
7. Byington CL, Hulten KG, Ampofo K, et al. Molecular Epidemiology of Pediatric Pneumococcal Empyema 2001-2007. J Clin Microbiol 2009; 16 de desembre [publicació en línia abans de la impressió].
8. Lin CJ, Chen PY, Huang FL, et al. Radiographic, clinical, and prognostic features of complicated and uncomplicated community-acquired lobar pneumonia in children. J Microbiol Immunol Infect 2006; 39: 489-495.
9. Tsigrelis C, Tleyjeh IM, Lahr BD, et al. Trends in invasive pneumococcal disease among older adults in Olmsted County, Minnesota. J Infect 2009; 59(3): 188-193.
10. Obando I, Muñoz-Almagro C, Arroyo LA. Pediatric parapneumonic empyema, Spain. Emerg Infect Dis 2008; 14: 1.390-1.397.
11. Sá-Leão R, Nunes S, Brito-Avô A, et al. Changes in pneumococcal serotypes and antibiotypes carried by vaccinated and unvaccinated day-care centre attendees in Portugal, a country with widespread use of the seven-valent pneumococcal conjugate vaccine. Clin Microbiol Infect 2009; 15(11): 1.002-1.007.
12. Huang SS, Hinrichsen VL, Stevenson AE, et al. Continued impact of pneumococcal conjugate vaccine on carriage in young children. Pediatrics 2009; 124(1): e1-11.
13. Trollfors B, Berg S, Backhaus E, et al. Invasive, paediatric, vaccine strains of Streptococcus pneumoniae: are there differences in clinical characteristics? Scand J Infect Dis 2009; 41(2): 84-87.
14. Hernandez-Bou S, Garcia-Garcia JJ, Esteva C, et al. Pediatric Parapneumonic Pleural Effusion: Epidemiology, Clinical Characteristics, and Microbiological Diagnosis. Pediatr Pulmonol 2009; 44(12): 1.192-1.200.
15. Muñoz-Almagro C, Esteva C, de Sevilla MF, et al. Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona. Infect 2009; 59(2): 75-82.
16. Leach AJ, Morris PS, McCallum GB, et al. Emerging pneumococcal carriage serotypes in a high-risk population receiving universal 7-valent pneumococcal conjugate vaccine and 23-valent polysaccharide vaccine since 2001. BMC Infect Dis 2009; 9: 121.
17. Song JH, Baek JY, Cheong HS et al. Changes of serotype and genotype in Streptococcus pneumoniae isolates from a Korean hospital in 2007. Diagn Microbiol Infect Dis 2009; 63(3): 271-278.
18. Richter SS, Heilmann KP, Dohrn CL, et al. Changing epidemiology of antimicrobial-resistant Streptococcus pneumoniae in the United States, 2004-2005. Clin Infect Dis 20091; 48(3): e23-33.
19. Mahjoub-Messai F, Doit C, Koeck JL, et al. Population snapshot of Streptococcus pneumoniae serotype 19A isolates before and after introduction of seven-valent pneumococcal Vaccination for French children. J Clin Microbiol 2009; 47(3): 837-840.
20. Techasaensiri C, Messina AF, Katz K, et al. Epidemiology and Evolution of Invasive Pneumococcal Disease Caused by Multidrug Resistant Serotypes of 19A in the 8 Years After Implementation of Pneumococcal Conjugate Vaccine Immunization in Dallas, Texas. Pediatr Infect Dis J 2009; 25 de novembre [publicació en línia abans de la impressió].
21. Dagan R, Givon-Lavi N, Leibovitz E. Introduction and Proliferation of Multidrug-Resistant Streptococcus pneumoniae Serotype 19A Clones That Cause Acute Otitis Media in an Unvaccinated Population. J Infect Dis 2009; 199: 776-785.
22. Bender JM, Ampofo K, Sheng X, et al. Parapneumonic empyema deaths during past century, Utah. Emerg Infect Dis 2009; 15(1): 44-48.
23. Li ST, Tancredi DJ. Empyema Hospitalizations Increased in US Children Despite Pneumococcal Conjugate Vaccine. Pediatrics 2009; 30 de novembre [publicació en línia abans de la impressió].
24. Chiappini E, Galli L, Bonsignori F, et al. Self-reported pediatricians' management of the well-appearing young child with fever without a source: first survey in an European country in the anti-pneumococcal vaccine era. BMC Public Health 2009; 9: 300.
25. PneumoADIP. Pneumococcal diseases. Disponible a: http://www.preventpneumo.org/diseases/pneumococcal_diseases/index.cfm [data de consulta: 22-12-09].

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Notes

Direct detection of *Streptococcus pneumoniae* in positive blood cultures by real-time polymerase chain reaction

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Abstract

We developed a real-time polymerase chain reaction specific for *Streptococcus pneumoniae* to be applied directly from blood culture bottles without previous DNA extraction step. For the 128 blood culture bottles tested, the assay had 94% and 98.4% sensitivity and specificity, respectively. This assay provides rapid and accurate identification of this pathogen.

Keywords: Real-time PCR; Blood culture bottles; *Streptococcus pneumoniae*

Streptococcus pneumoniae is one of the major bacterial pathogens worldwide, causing bacteremia and community-acquired pneumonia (Johnson et al., 2008). According to the World Health Organization (WHO) (2008), an estimated 700 000 to 1 million children younger than 5 years die of pneumococcal disease every year, and this represents a major global public health problem.

Detection of the pathogen with automated blood culture systems and subsequent identification with biochemical tests takes a minimum of 48 h (Lakshmi, 2001). Rapid detection of pathogens in blood from septic patients may allow a reduction in the unnecessary use of broad-spectrum antimicrobials and should prevent further emergence of resistance (Gebert et al., 2008; Paule et al., 2005).

The aims of this study were to minimize the waiting time between the detection of a positive blood culture and pathogen identification by microbiologic molecular techniques and to test the sensitivity and specificity of a homemade real-time polymerase chain reaction (PCR) assay for *S. pneumoniae* in blood culture samples without previous DNA extraction step.

We studied a total of 128 blood culture bottle samples (60 positive blood cultures and 68 negative blood cultures) from 128 pediatric patients who attended at the University Hospital Sant Joan de Déu (Esplugues) in Barcelona, Spain, from January to December 2007 and from March to April 2009. This hospital is a tertiary-care children's and maternity hospital with 345 beds and an average referral population of 210 000 children younger than 18 years. Blood samples were inoculated into FAN-aerobic bottles (BioMérieux Laboratories, Boxtel, The Netherlands) containing an antimicrobial-absorbent resin. The bottles were cultured immediately using the automatic Bact-Alert system (BioMérieux Laboratories) for 5 days at 37 °C. All positive blood culture bottles were identified by conventional biochemical and serologic techniques.

Samples were collected directly from the blood culture bottles after incubation (by signal-positive growth or by negative detection after 5 days). An aliquot of 1.5 mL was taken from the bottle, and then the sample was briefly centrifuged (6000 × *g* for 1 min). Five microliters of the aqueous supernatant was used directly in real-time PCR.

The homemade real-time PCR assay coamplified a specific sequence of *S. pneumoniae* in the pneumolysin gene (*ply*) (GenBank accession number X52474) and RNase P human gene as internal control. The master mixture contained 12.5 μL of TaqMan® Universal PCR Master Mix

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(Applied Biosystems Foster City, CA), 0.9 μmol/L final concentration of *S. pneumoniae* specific primers (*ply*-F: 5′-TGCAGAGCGTCCTTTGGTCTAT-3′ and *ply*-R: 5′-CTC TTACTCGTGGTTTCCAACTTGA-3′), 0.3 μmol/L final concentration of hybridization probe, 1 μL of TaqMan® RNase P Detection Reagents (VIC™) (Applied Biosystems), and 1.20 μL of PCR water. Twenty-microliter aliquots of master mixture were previously prepared and stored at −20 °C for subsequent use. The cost of each PCR reaction was less than 2 Euros.

Real-time PCR assays were carried out in a final 25.5-μL reaction volume, including 20 μL of prepared master mixture and 5 μL of sample. An additional 0.5 μL of Human DNA male (Applied Biosystems) was added to each tube to test potential inhibition.

DNA was amplified with the Applied Biosystems 7300 Real-time PCR System (Applied Biosystems) using the following cycling parameters: 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min (total time, 1 h 59 min). Amplification data were analyzed by instrument software (SDS, Applied Biosystems). Negative results were defined as those with cycle threshold (C_T) values above 40.

For testing the lowest limit of detection of the assay, 0.5-McFarland suspension was made from an *S. pneumoniae* strain obtained from our bacteriologic laboratory, and serial dilutions from 1.7 ng/μL (7.107 copies/mL) to 0.6 fg/μL (7 copies/mL) were performed. The real-time PCR assay correctly detected all serial dilutions (range, 7–7.107 copies/mL).

From the total of 60 positive blood culture bottles, 57 types of bacteria were identified by culture: 48 Gram-positive cocci, including 18 *S. pneumoniae*, 3 *Streptococcus agalactiae*, 8 *Streptococcus mitis*, 4 *Streptococcus viridans* group, 3 *Enterococcus faecalis*, 1 *Enterococcus faecium*, 1 *Micrococcus* spp., 1 *Staphylococcus aureus*, 1 *Staphylococcus epidermidis*, 2 *Staphylococcus simulans*, 4 plasma coagulase-negative *Staphylococcus*, and 1 *Corynebacterium* spp.; 8 Gram-negative bacilli, including 4 *Escherichia coli*, 1 *Acinetobacter lwoffii*, 1 *Enterobacter cloacae*, 1 *Klebsiella pneumoniae*, and 1 enteric salmonella; and 1 Gram-negative cocci corresponding to 1 *Neisseria meningitidis*. Any pathogen found was isolated by culture in 2 bottles with Gram-positive cocci and 1 with Gram-negative bacilli.

The results of the comparative microbiologic study of the culture and the *ply* real-time PCR in blood cultures are shown in Table 1. Comparison of real-time PCR showed that 17 of 18 *S. pneumoniae* had a positive result for real-time PCR, with a C_T <28. For the sample that was only identified by culture, the real-time PCR was inhibited with no detection of RNAse P internal control. One of the 8 *S. mitis* isolates had a positive result for the PCR, so we have a false-positive result. From the 2 samples that were Gram-positive cocci but without any pathogen isolated in blood culture, the *ply* real-time PCR was positive in one of them. The rest of the samples with other pathogens and all 68 negative blood cultures were negative by real-time PCR. The sensitivity and specificity of real-time PCR, with culture as the gold standard, were 94% and 98.4%, respectively.

Table 1
Comparative microbiologic study of culture and real-time PCR in blood cultures of 128 patients

Gram stain result	Pathogen isolated in blood culture	No. of patients	Real-time PCR result	
			Positive	Negative
GPC	*S. pneumoniae*	18	17	1
GPC	*S. agalactiae*	3	0	3
GPC	*S. mitis*	8	1	7
GPC	*S. viridans* group	4	0	4
GPC	*E. faecalis*	3	0	3
GPC	*E. faecium*	1	0	1
GPC	*Micrococcus* spp.	1	0	1
GPC	*S. aureus*	1	0	1
GPC	*Staphylococcus auricularis*	1	0	1
GPC	*S. epidermidis*	1	0	1
GPC	*S. simulans*	2	0	2
GPC	Coagulase-negative staphylococci	4	0	4
GPC	*Corynebacterium* spp.	1	0	1
GPC	Non detected	2	1	1
GNB	*E. coli*	4	0	4
GNB	*A. lwoffii*	1	0	1
GNB	*E. cloacae*	1	0	1
GNB	*K. pneumoniae*	1	0	1
GNB	Enteric salmonella	1	0	1
GNB	*Non detected*	1	0	1
GNC	*N. meningitidis* group B	1	0	1
Negative	Nondetected	68	0	68
	Total	128	19	109

Sensitivity and specificity of *pneumolysin* real-time PCR according to culture results: 94% and 98.4%, respectively.
GPC = Gram-positive cocci; GNB = Gram-negative bacilli; GNC = Gram-negative cocci.

S. pneumoniae, as one of the major bacterial pathogens worldwide, requires a fast early diagnostic test (Johannes, 2008; WHO, 2008). Like several authors (Gebert et al., 2008; Gröbner and Kempf, 2007; Hogg et al., 2008; Kurupati et al., 2004; Paule et al., 2005; Selvarangan et al., 2003), we found high sensitivity and specificity for the rapid identification of 1 invasive pathogen using molecular technology in comparison with conventional identification from blood cultures.

Of note, some of these authors (Gebert et al., 2008; Hogg et al., 2008) evaluated and compared different extraction methods and highlighted the importance of evaluating specific DNA extraction methods. However, we tested the technology without previous DNA extraction. The result is a rapid, specific, and sensitive *S. pneumoniae* detection test (it takes less than 2 1/2 h) because the master mixture is previously prepared and no extraction method is required. The bottles used for bacterial culture have FAN medium that contains adsorbent material called Ecosorb (containing adsorbent charcoal, fuller's earth, and other components). Activated charcoal is an extremely porous carbonaceous

adsorbent material with a very large surface area available for adsorption, which allows the removal of PCR inhibitors from the sample. Abolmaaty et al. (2007) reported the use of activated charcoal as a promising and convenient technology for removal of PCR inhibitors from biologic samples.

We used *pneumolysin* gene as the target for *S. pneumoniae* detection, which has been successfully used for the diagnosis of pneumococcal infection (Lahti et al., 2006). However, the results of the present study show that 1 nonpneumococcal *Streptococcus*, *S. mitis*, had a positive result for the real-time PCR. According to several authors (Carvalho et al., 2007; Kee et al., 2008), the presence of *ply* gene in other streptococci, in particular, *S. mitis*, could be a limitation of this assay, and other primers for detecting *S. pneumoniae* by PCR, such as *autolysin* (*lytA*) gene, could have higher specificity.

In this study, we examined the useful contribution of molecular techniques to the microbiologic identification of *S. pneumoniae*, but in the future, it may become possible to detect more pathogens, not only for pathogen identification but also for resistance, serogrouping (Munoz-Almagro et al., 2008), and serotyping assays (Tarrago et al., 2008). This method may prove to be suitable for implementation in routine emergency diagnostic laboratories.

In conclusion, real-time PCR can be used for rapid, accurate detection of *S. pneumoniae* bacteremia, which can permit optimal therapeutic treatment at the earliest time. It offers the benefit of yielding reliable results in a few hours' time as opposed to the days required with conventional methods. PCR technology has also shown its great potential in routine molecular diagnostic screening for the identification of several pathogens.

References

Abolmaaty A, Gu W, Witkowsky R, Levin RE (2007) The use of activated charcoal for the removal of PCR inhibitors from oyster samples. *J Microbiol Methods* 68:349–352.

Carvalho MGS, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS (2007) Evaluation and improvement of real-time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. *J Clin Microbiol* 45:2460–2466.

Gebert S, Siegel D, Wellinghausen N (2008) Rapid detection of pathogens in blood culture bottles by real-time PCR in conjunction with the pre-analytic tool MolYsis. *J Infect* 10:1–10.

Gröbner S, Kempf VA (2007) Rapid detection of methicillin-resistant staphylococci by real-time PCR directly from positive blood culture bottles. *Eur J Clin Microbiol Infect Dis* 26:751–754.

Hogg GM, McKenna JP, Ong G (2008) Rapid detection of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* directly from positive BacT/Alert blood culture bottles using real-time polymerase chain reaction: evaluation and comparison of 4 DNA extraction methods. *Diagn Microbiol Infect Dis* 61:446–452.

Johannes RS (2008) Epidemiology of early-onset bloodstream infection and implications for treatment. *Am J Infect Control* 36(S171): e13–e17.

Johnson AW, Osinusi K, Aderele WI, Gbadero DA, Olaleye OD, Adeyemi-Doro FA (2008) Etiologic agents and outcome determinants of community-acquired pneumonia in urban children: a hospital-based study. *J Natl Med Assoc* 100:370–385.

Kee C, Palladino S, Kay I, Pryce TM, Murray R, Rello J, Gallego M, Lujan M, Muñoz-Almagro C, Waterer GW (2008) Feasibility of real-time polymerase chain reaction in whole blood to identify *Streptococcus pneumoniae* in patients with community-acquired pneumonia. *Diagn Microbiol Infect Dis* 61:72–75.

Kurupati P, Chow C, Kumarasinghe G, Poh CL (2004) Rapid detection of *Klebsiella pneumoniae* from blood culture bottles by real-time PCR. *J Clin Microbiol* 42:1337–1340.

Lakshmi V (2001) Culture of body fluids using the Bact/Alert system. *Indian J Med Microbiol* 19:44–50.

Lahti E, Mertsola J, Kontiokari T, Eerola E, Ruuskanen O, Jalava J (2006) Pneumolysin polymerase chain reaction for diagnosis of pneumococcal pneumoniae and empyema in children. *Eur J Clin Microbiol Infect Dis* 25:783–789.

Munoz-Almagro C, Rodriguez-Plata MT, Marin S, Esteva C, Esteban E, Gene A, Gelabert G, Jordan I (2008) Polymerase chain reaction for diagnosis and serogrouping of meningococcal disease in children. *Diagn Microbiol Infect Dis* (Epub ahead of print).

Paule SM, Pasquariello AC, Thomson Jr RB, Kaul KL, Peterson LR (2005) Real-time PCR can rapidly detect methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* directly from positive blood culture bottles. *Am J Clin Pathol* 124:404–407.

Selvarangan R, Bui U, Limaye AP, Cookson BT (2003) Rapid identification of commonly encountered *Candida* species directly from blood culture bottles. *J Clin Microbiol* 41:5660–5664.

Tarrago D, Fenoll A, Sanchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C, Hausdorff WP, Casal J, Obando I (2008) Identification of pneumococcal serotypes from culture-negative clinical specimens by novel real-time PCR. *Clin Microbiol Infect* 14:828–834.

World Health Organization (WHO) (2008) New and under-utilized vaccines implementation (NUVI). Available at: http://www.who.int/nuvi/pneumococcus/en/. Accessed October 22.

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Emergence of invasive pneumococcal disease caused by multidrug-resistant serotype 19A among children in Barcelona☆

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Summary *Objective:* To describe the epidemiology of invasive pneumococcal disease (**IPD**) caused by *Streptococcus pneumoniae* serotype 19A.
Methods: We studied all children and adolescents with **IPD** caused by serotype 19A who were admitted to a Children's Hospital in Barcelona (1997—2007). Serotyping, antibiotic susceptibility and clonal analysis were performed.
Results: Comparing the pre-vaccine period (1997—2001) with the early vaccine period (2002—2004) and the late vaccine period (2005—2007) there was an increase of **IPD** caused by serotype 19A: 1 of 58 episodes (1.7%) vs. 8 of 54 episodes (14.8%) vs. 27 of 123 episodes (21.9%), respectively ($P = 0.002$). All *S. pneumoniae* serotype 19A isolated in the pre-vaccine and early vaccine periods ($n = 9$) were penicillin susceptible, while in the late vaccine period, 12 of 27 (44%) were penicillin nonsusceptible ($P = 0.01$).

A clonal analysis revealed 15 different sequence types (STs) expressing serotype 19A. 10 of them were preexisting STs associated with serotype 19A including the multidrug-resistant ST320 and ST276.
Conclusion: There was an increase of **IPD** caused by *S. pneumoniae* serotype 19A which was mainly related with the emergence of preexisting clones several of them closely related with

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international multidrug-resistant clones. These results should be considered when selecting the new conjugate pneumococcal vaccines.

Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, especially among young children. There are 91 different pneumococcal serotypes that can be distinguished by their polysaccharide capsule, but only around one third produces invasive pneumococcal disease (**IPD**).[1]

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. In Spain **PCV7** was introduced in June 2001 and the current estimated vaccination uptake is around 50%.[2] In pre-vaccine years the **PCV7** serotypes were responsible for 50—80% of all **IPD** varying widely depending on geographic location.[1] In addition, **PCV7** serotypes were often penicillin and multidrug-resistant isolates. Following introduction of **PCV7** in the USA there was a dramatic decline in **IPD** rates and drug-resistant pneumococci.[3,4] However, recent reports from the USA and other countries have shown an emergence of **IPD** caused by non-**PCV7** serotypes[5,6] and the increase of drug-resistant pneumococcal clones expressing non-**PCV7** serotypes in nasopharyngeal carriage and invasive isolates.[7—10]

It has been shown that **PCV7** protects against pneumococcal nasopharyngeal colonization by vaccine serotypes.[11] Therefore, the risk of colonization and consequently the risk of **IPD** by **PCV7** serotypes decrease both in the vaccine target age group (young children) and in older children and adults because of herd immunity.[12]

Reducing carriage of **PCV7** serotypes may produce a vacant ecological niche which may be filled by non-**PCV7** serotypes, and this phenomenon is called ''serotype replacement''. Replacement colonization has been reported,[13] but little is know about the relevance of serotype replacement in the epidemiology and clinical findings of **IPD**.

Recently, some reports have shown that serotype 19A is becoming one important cause of pneumococcal disease in the USA population with high vaccine uptake.[5,14—16] However, it has also been reported an increase of pneumococcal disease caused by serotype 19A in Korea and Israel[17,18] where PCV7 is not used which suggests that other non-vaccine factors such as antibiotic consumption may play a role in this increase. It is worrisome that some of these serotype 19A pneumocci are multidrug-resistant isolates.[19]

The aim of this study was to describe the clinical and molecular epidemiology of *S. pneumoniae* serotype 19A causing **IPD** among children in Barcelona.

Patients and methods

Patients and setting

We studied all children and adolescents with invasive pneumococcal disease **(IPD)** who were admitted to Sant Joan de Deu Hospital in Barcelona (January 1997 to December 2007), and we selected for the present study those infected with serotype 19A. A detailed description of our institution and the geographic area was reported elsewhere.[2] The Clinical Microbiology Laboratory monitored all culture-proven pneumococcal infections and several variables are routinely registered including demographic data, identification hospital number, type of infection, and antimicrobial susceptibility. Serotyping, antimicrobial susceptibility testing and clonal analysis by multilocus sequence typing (MLST) were performed as previously described,[2] and summarized below.

PCV7 is not currently subsidized by the Spanish Health Service. **PCV7** uptake has increased since its introduction in June 2001 with an estimated **PCV7** coverage in the year 2007 about 45—50%.[2]

Definitions

Invasive pneumococcal disease (**IPD**) was defined as the presence of clinical findings of infection together with isolation of *S. pneumoniae* in blood, cerebrospinal fluid or any other sterile fluid by culture. No other microbiological techniques, such as polymerase chain reaction (PCR), were used for the diagnosis of **IPD**.

IPD was classified according to the International Classification of Disease, Ninth Revision (ICD-9) specific for diseases caused by *S. pneumoniae* including: meningitis, pneumonia, parapneumonic empyema, occult bacteremia, sepsis, arthritis, peritonitis, and endophthalmitis. We reviewed the electronical medical records and registered demographic and clinical variables including: age, sex, date of admission, clinical manifestations, outcomes and vaccination status. Data were recorded following the guidelines of the Hospital's Ethical Committee.

Serotyping and antimicrobial susceptibility

All isolates were serotyped by Quellung reaction. In addition, isolates identified as serogroup 19 during the pre-vaccine period were also tested by specific Real Time PCR of serotype 19A according to a published assay.[20]

Agar dilution technique was used to determine the minimal inhibitory concentrations (MICs) of penicillin and other antibiotics. Antibiotic susceptibility was defined according to the 2008 breakpoints by the Clinical Laboratory Standards Institute.[21] Isolates with intermediate or high level resistance were defined as nonsusceptible. Multidrug resistance was defined as nonsusceptible to three or more antimicrobial agents.

Clonal analysis

MLST was performed as reported elsewhere.[22] The assignment of alleles and sequence types (ST) were carried out using the software at the pneumococcal web page:

Table 1 Invasive Pneumococcal Disease (**IPD**) caused by serotype 19A in three periods (pre-vaccine period, early vaccine period, and late vaccine period).

	Serotype 19A episodes/Total episodes (%)			
	Pre-vaccine (1997–2001)	Early vaccine (2002–2004)	Late vaccine (2005–2007)	*P* value[a]
Overall episodes	1/58 (1.7)	8/54 (14.8)	27/123 (21.9)	0.002
Clinical manifestations				
- Meningitis	0/18 (0)	1/8 (12.5)	4/24 (16.6)	0.198
- Pneumonia (overall)	1/15 (6.6)	1/22 (4.5)	11/58 (18.9)	0.169
without empyema	1/8	0/9	5/24	
with empyema	0/7	1/13	6/34	
- Bacteremia/sepsis	0/17 (0)	6/16 (37.5)	9/32 (28.1)	0.024
- Others[b]	0/8 (0)	0/8	3/9 (33.3)	0.048

[a] Chi-square test (two by three tables).
[b] Others (arthritis, appendicitis, and endophthalmitis).

www.mlst.net. Analysis of ST and assignment to clonal complex was performed with the eBURST program.[23] STs that shared five of seven allelic identities (double locus variants [DLV]) or shared six of seven allelic (single locus variants [SLV]) were considered a clonal complex.

Statistical analysis

The study period was divided into three periods: the pre-vaccine period (1997–2001), the early vaccine period (2002–2004) and the late vaccine period (2005–2007). We used the Chi-square test or Fisher's exact test, when appropriated, to compare proportions, and Student *t*-test to compare means. Statistical analyses were performed using SPSS for windows, version 14.0. Rates of **IPD** (episodes/100,000 population) were calculated using children population in the southern Barcelona area as reported elsewhere.[2] Two-sided *P* values $\leq$0.05 were considered statistically significant.

Results

During the 11-year study period, there were 235 episodes of invasive pneumococcal disease (**IPD)** occurring in 230 children; the mean age was 3.1 years (range 1 month–17 years) and 60% were males. Overall, there were 35 different serotypes, and serotype 19A was recovered from 15.3% of the episodes (36 of 235 isolates).

Comparing the pre-vaccine period (1997–2001) with the early vaccine period (2002–2004) and the late vaccine period (2005–2007) there was a significant

Table 2 Characteristics of patients with invasive pneumococcal disease (**IPD**) caused by serotype 19A vs. other serotypes.

	Episodes (%) Serotype 19A (n = 36)	Episodes (%) Other serotypes (n = 199)	*P* value
Age (yrs) (±SD)	1.8 (±2.2)	3.4(±3.4)	**0.01**
Sex (males)	23 (63.9)	119 (56.7)	**0.64**
Underlying conditions	1 (2.8)[a]	19 (9.5)[b]	**0.18**
PCV7 vaccination[c]	3 (9.1)	20 (11)	**0.73**
Clinical Manifestations of IPD			
- Meningitis	5 (13.9)	45 (22.6)	**0.23**
- Pneumonia (overall)[d]	13 (36)	82 (41.2)	**0.56**
with empyema	7	47	
without empyema	6	35	
- Bacteremia/sepsis	15 (41.7)	50 (25.1)	**0.04**
- Others	3 (8.3)	22 (11)	**0.62**
PICU admission	4 (11.1)	49 (24.6)	**0.07**
Days of hospital stay (mean±SD)	9.2 (±6.8)	12.1(±9.1)	**0.07**
Mortality	1 (2.8)	8 (4.1)	**0.7**

PICU: pediatric intensive care unit.
Statistical methods: Chi-square test (categorical variables) and Student *t*-test (continuous variables).
[a] A child with chronic pulmonary disease.
[b] Includes 8 IPD episodes in 7 children with malignant disease and innmunosupressive therapy, 5 IPD episodes in 2 children with CSF leakage, 2 with HIV infection, 1 with chronic pulmonary disease, 2 with chronic cardiac disease, and 1 with chronic renal failure.
[c] The PCV7 vaccinations status was available in 214 (33 with serotype 19A and 181 with others serotypes).
[d] Pneumonia with positive blood and/or pleural fluid cultures.

increase of **IPD** caused by serotype 19A: 1 of 58 episodes (1.7%) vs 8 of 54 episodes (14.8%) vs 27 of 123 episodes (21.9%), respectively ($P = 0.002$). In terms of rates of **IPD** per 1000 blood cultures performed, serotype 19A also increased (from 0.04 episodes per 1000 blood cultures during pre-vaccine period to 0.48 during early vaccine period and 1.39 during late vaccine period; $P < 0.001$). Among children <5 years, rates of **IPD** caused by serotype 19A, comparing early vaccine period and late vaccine period, increased 147%; 95% CI, 11−448% (from 4.8 to 11.9 episodes per 100,000 population, $P = 0.02$). Table 1 shows the clinical manifestations of **IPD** in the three periods. Although the numbers were small, a statically significant increase was observed for bacteremia/sepsis and other infection group.

IPD caused by serotype 19A compared with other serotypes

As shown in Table 2, children with **IPD** caused by serotype 19A were younger than those infected with other serotypes: mean age of 1.8 years (range 1 month−11 years) vs 3.4 years (range 1 month−17 years), respectively, $P = 0.01$. Regarding the clinical manifestations, bacteremia/sepsis was more frequently found in the group of patients infected by serotypes 19A ($P = 0.04$). There were no statistically significant differences in underlying conditions (defined according to the criteria of the American Academy of Pediatrics),[24] vaccination uptake with PCV7 and mortality. Although it did not reach statistical significance the PICU admission rate and length of hospital stay tended to be greater in the other serotypes group.

Emergence of drug-resistant serotype 19A

Table 3 shows antibiotic resistance including CLSI (Clinical laboratory Standard Institute) meningeal and nonmeningeal breakpoints. According to meningeal breakpoints, in the pre-vaccine and the early vaccine periods, all serotype 19A isolates ($n = 9$) were penicillin susceptible, whereas in the late vaccine period 12 of 27 isolates (44%) were penicillin nonsusceptible ($P = 0.01$) (Table 2); 8 of these 12 isolates (66.6%) showed multidrug resistance (defined as nonsusceptible to three or more antimicrobial agents). Of note, 3 of the 8 multidrug-resistant isolates had a cefotaxime MIC of 2 μg/mL and were fully resistant to penicillin, erythromycin and tetracycline; these 3 isolates belonged to ST320 or ST276 clones (see below).

Molecular analysis of serotype 19A isolates

Of the 36 isolates, 35 (97%) were available for molecular analysis (Fig. 1). Overall, there were 15 different sequence types (STs) expressing serotype 19A: 10 preexisting STs associated with serotype 19A including the multidrug-resistant ST320 and ST276; 2 preexisting STs (ST30 and ST1793) not previously associated with serotype 19A; and 3 new STs (ST2589, ST2618 and ST3438).

Among the 24 penicillin susceptible 19A isolates, ST1201 was detected in 10 isolates. A clonal group identified by eBURST (that include ST199 as the primary founder of the group) was detected in another 8 isolates, including ST416, ST450, ST274, ST199, and the new ST2618. Clonal compositions of the remaining 6 isolates were: ST202, ST2589, ST1793, and ST30 (Fig. 1).

The analysis of the 12 penicillin nonsusceptible 19A isolates, revealed that of all them have identical allelic profiles or are single locus variants or double locus variants of several resistant international clones included in the PMEN (pneumococcal molecular epidemiology network) (Table 4). Thus, we detected the well-known Spain^{23F}-1 (ST81) in 2 isolates and the multiresistant clone ST320 which is a DLV of Taiwan^{19F}-14 in 2 isolates. In addition, there were 3 unusual clones: a single locus variant of Denmark^{14}-32 (ST276) in 4 isolates, a double locus variant of the same clone Denmark^{14}-32 (ST2013) in 3 isolates and one additional strain with a new MLST profile (ST3438) that was submitted to the curator of MLST for designation which was detected as a DLV of Columbia^{23F}-26. Table 4 shows detailed information of the clinical manifestations, clonal composition, and antimicrobial susceptibility patterns.

Table 3 Antimicrobial susceptibility of serotype 19 A pneumococcal strains ($n = 36$).

MIC (mg/L)	Pre-vaccine and early vaccine period	Late vaccine period	*P* Value
	No. of strains (%) $n = 9$	No. of strains (%) $n = 27$	
Penicillin G (meningeal breakpoints)			
≤0.06	9 (100)	15 (56)	0.01
≥0.12	0	12 (44)	
Penicillin G (nonmeningeal breakpoints)			
≤2	9 (100)	26 (96.3)	0.5
4	0	1 (3.7)	
≥8	0	0	
Cefotaxime (meningeal breakpoints)			
≤0.5	9 (100)	22 (82)	0.4
1	0	3 (11)	
≥2	0	2 (7)	
Cefotaxime (nonmeningeal breakpoints)			
≤1	9 (100)	25 (92.6)	0.4
2	0	2 (7.4)	
≥4	0		
Erythromycin			
≤0.25	3 (33)	18 (66.6)	0.07
0.5	1 (11)	0	
≥1	5 (56)	9 (33.3)	
Tetracycline			
≤2	3 (33.3)	14 (57.8)	0.4
4	0	1 (3.7)	
≥8	6 (66.6)	12 (40.5)	
Chloramphenicol			
≤4	7 (78)	25 (93)	0.2
≥8	2 (22)	2 (7)	

Statistical Methods: Chi-square test (2 by k tables).

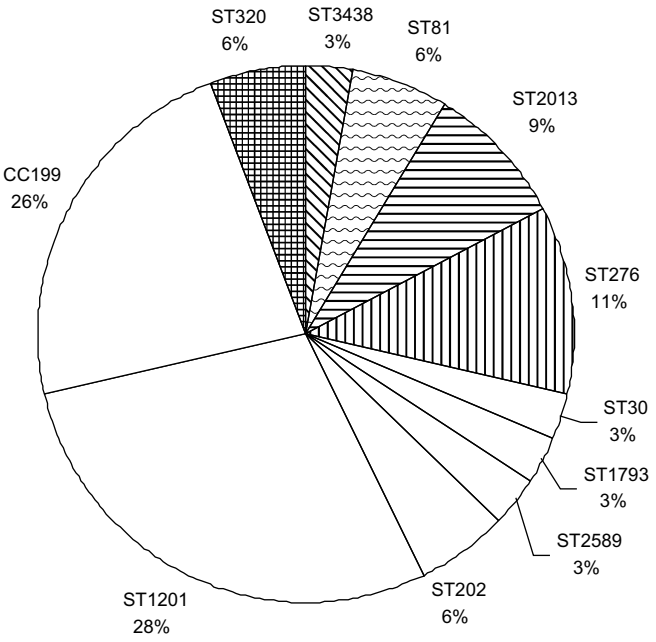

Figure 1 Clonal composition of 35 *Streptococcus pneumoniae* strains of serotype 19A isolated from children with invasive pneumococcal disease (**IPD**). Penicillin nonsusceptible strains are marked with grid. Clonal complex with ST199 as founder (CC199) includes ST199, ST416, ST274, ST2618 and ST450.

Discussion

The implementation of **PCV7** for children has been associated with a decline in invasive pneumococcal disease (**IPD**)[3,4] and nasopharyngeal colonization rates[11] caused by vaccine serotypes. An important question is whether or not the non-vaccine serotypes (or which of these serotypes) reach an advantage to colonize the nasopharynx and/or to produce **IPD** (the called replacement phenomenon). It seams reasonable to think that if this replacement occurs it could be especially significant for non-vaccine serotypes that were common inhabitants of the nasopharynx and/or had a high potential to produce outbreaks and were widely disseminated in the community before PCV7.

Recently an increase of **IPD** caused by non-**PCV7** serotypes in USA and Spain has been described.[2,5,6,16,25] At least two different models could explain the emergence of these non-vaccine serotypes.

First, the overgrowth of some non-vaccine serotypes, such as serotypes 1 and 5, which have a homogeneous clonal composition and are rarely isolated in healthy nasopharyngeal carriers. However, these serotypes (serotype 1 and 5) have a high potential to cause **IPD** and may produce outbreaks, particularly in a localized geographical area.[26]

Second, the enhancement of some non-vaccine serotypes, such as serotype 19A, which have a diverse clonal composition and are often isolated from healthy nasopharyngeal carriers. These serotypes (like serotype 19A) can produce **IPD** and their dissemination in the community could be expected to become a generalized phenomenon.

The present study shows an increase of **IPD** caused by serotype 19A, and a special concern about the emergence of serotype 19A variants of internationally multiresistant clones of PMEN. Recent reports have documented an increase of antibiotic resistance among non-vaccine pneumococcal serotypes such as serotype 19A.[14,27] This may occur by different mechanisms: 1) capsular switching from an antibiotic resistant clone expressing a vaccine serotype in the pre-vaccine era that express a non-vaccine serotype in the vaccine era. It has been observed in serotype 19A variants circulating in USA[28]; 2) by emergence of minor antibiotic resistant clones existing prior to vaccination; and 3) the appearance of new clones.

Although we observed the three mechanisms in our study, the most important was the emergence of unusual antibiotic resistant clones expressing serotype 19A that had been considered as a minor cause of **IPD** in the pre-vaccine era.

In brief, the international multiresistant clone Spain^{23F}-1 (ST81) was widely encountered in Spain and other countries in the pre-vaccine era,[29,30] but it was expressing vaccine serotypes, mainly serotype 23F, while in the present study we detected this clone expressing serotype 19A. Also we observed an emergence of minor clones existing in the pre-vaccine era such as ST276 reported in Portugal,[31] France and Turkey (www.mlst.net) or ST2013 reported in Egypt according also the web page of MLST. Of note, the multiresistant clone ST320 had been previously reported in Korea,[17] Australia and Norway,[32] and recently identified in USA[15,33] which aroused special concern.

The capsule of pneumococci is a major virulence factor and this may explain why certain serotypes have greater potential to cause **IPD**.[34] Up to date, there is a controversy about the impact of clonal type on the invasive disease potential of pneumococci.[34,35] We do not know if the minor clones found in the present study may continue with an international spread or they could be sporadic cases. Interestingly, the first highly penicillin resistant clones isolated in 1977 were

Table 4 Characteristics of patients, clonal compostition and antimicrobial susceptibility in 36 IPD episodes caused by serotype 19A.

Pt	Months	Sex	Year of isolation	Prior PCV7	Prior antibiotic therapy	Clinical manifestation	ST	PMEN clone	PEN MIC	CTX MIC	CHL MIC	ERY MIC	TET MIC
1	96	M	2001	Non	AZY	Pneumonia	202	DLV-Taiwan^{19F}-14	0.015	0.03	4	0.5	64
2	12	M	2002	Non	AMX/CLV	Bacteremia/sepsis	30[a]	non-related	0.015	0.015	16	>128	16
3	6	M	2002	N.A	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	16	>128	64
4	24	M	2002	N.A	CLA	Empyema	2589[b]	non-related	0.015	0.03	4	>128	32
5	7	F	2002	Non	Non	Bacteremia/sepsis	N.A	non available data	0.015	0.03	4	64	32
6	18	M	2003	N.A	Non	Bacteremia/sepsis	416	DLV Netherlands^{15B}-37	0.030	0.015	4	0.12	0.25
7	12	F	2003	Yes	Non	Meningitis	1793[a]	TLV-Netherlands^{14}-35	0.015	0.015	4	0.12	0.25
8	12	M	2004	Non	Non	Bacteremia/sepsis	202	DLV-Taiwan^{19F}-14	0.03	0.03	4	128	32
9	1	F	2004	Non	Non	Bacteremia/sepsis	1201	non-related	0.03	0.015	4	0.12	0.50
11	12	F	2005	Non	Non	Empyema	81	Spain^{23F}-1	1	0.5	4	0.25	8
10	6	M	2005	Non	Non	Meningitis	81	Spain^{23F}-1	0.50	0.25	8	0.12	8
12	1	M	2005	Non	Non	Bacteremia/sepsis	199	Netherlands^{15B}-37	0.015	0.015	4	0.12	0.25
14	72	M	2005	Non	Non	Arthritis	276	Denmark^{14}-32	2	2	4	128	16
15	6	M	2005	Non	AMX/CLV	Bacteremia/sepsis	276	Denmark^{14}-32	0.5	0.5	4	128	8
13	24	M	2005	Non	Non	Empyema	276	Denmark^{14}-32	1	1	4	128	16
18	24	M	2005	Yes	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	16	128	64
16	8	M	2005	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
17	48	M	2005	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
19	11	M	2006	Non	Non	Bacteremia/sepsis	199	Netherlands^{15B}-37	0.015	0.015	4	0.12	0.25
20	10	M	2006	Non	Non	Bacteremia/sepsis	274	SLV Netherlands1^{5B}-37	0.015	0.015	4	128	32
21	10	F	2006	Non	Non	Bacteremia/sepsis	276	Denmark^{14}-32	1	0.5	4	128	32
22	12	F	2006	Non	Non	Empyema	416	DLV Netherlands^{15B}-37	0.015	0.015	4	0.12	0.25
23	24	F	2006	Non	Non	Pneumonia	1201	non-related	0.015	0.015	4	0.12	0.25
24	24	F	2006	Non	Non	Empyema	2013	DLV-Denmark^{14}-32	0.25	0.12	4	0.12	64
25	36	M	2006	Non	Non	Empyema	2013	DLV-Denmark^{14}-32	0.5	0.12	4	0.12	32
26	6	M	2006	Non	Non	Meningitis	2618[b]	TLV Netherlands^{15B}-37	0.03	0.015	4	0.12	0.25
27	24	F	2006	Non	Non	Arthritis	3438[b]	DLV columbia^{23F}-26	0.5	0.12	4	0.12	0.25
28	25	M	2007	Non	CEF	Empyema	320	DLV-Taiwan^{19F}-14	4	2	4	128	32
29	132	F	2007	Non	Non	Meningitis	416	DLV Netherlands^{15B}-37	0.015	0.015	4	0.12	0.25
30	6	F	2007	Yes	Non	Pneumonia	450	DLV Netherlands^{15B}-37	0.015	0.015	4	0.12	0.25
31	16	F	2007	Non	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	4	0.12	0.25
32	12	F	2007	Non	Non	Bacteremia/sepsis	1201	non-related	0.015	0.015	4	0.12	0.25
33	3	M	2007	N.A	Non	Meningitis	1201	non-related	0.015	0.015	4	0.12	0.25
34	11	M	2007	Non	CLA	Pneumonia	2013	DLV-Denmark^{14}-32	1	1	4	128	4
35	17	M	2007	Non	Non	Bacteremia/sepsis	320	DLV-Taiwan^{19F}-14	2	1	4	128	32
36	15	M	2007	Non	Non	Arthritis	1201	non-related	0.015	0.015	4	0.12	0.25

Abbreviations: M, Male; F, Female; PEN, penicillin; AMX/CLV, amoxicillin/clavulanic; CEF, cefuroxime; CTX, cefotaxime; CHL, choramphenicol; ERY, erythromycin; AZY, azithromycin; CLA,claritromicin; TET, tetracycline; MIC, minimun inhibitory concentration (μg/ml).

A summary of clonal analysis of 14 of these 36 serotype 19A strains had been included in a previous report.[2]

[a] These sequence types have only been associated with serotypes other than 19A.

[b] New sequence types that were identified in this study and deposited in the international multilocus sequence type database.

associated with serotypes 14 and 19A but international spread was more common for serotype 14.[36]

In conclusion, our study and others[14,15,19,27,33] show that *S. pneumoniae* serotype 19A is spreading rapidly and is becoming one important cause of **IPD** in the **PCV7** era. The spread of serotype 19A was related with the emergence of multiple penicillin susceptible and nonsusceptible clones, several of them closely related with well-known international multiresistant clones.

The possibility that the emergence of serotype 19A causing IPD is co-incidental and not related to vaccination should be considered. However, based on the reported experience and in our own data one might think that the vaccine is at least an additional factor that may contribute in selecting the most successfully serotypes for global spreading.

Continued surveillance of pneumococcal serotypes causing **IPD** is mandatory for the new conjugate vaccine strategies.

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References

1. Hausdorff WP, Bryant J, Paradiso PR, Siber GR. Which pneumococcal serogroups cause the most invasive disease:implications for conjugate vaccine formulation and use, part 1. *Clin Infect Dis* 2000;**30**:100—21.
2. Muñoz-Almagro C, Jordan I, Gene A, Latorre C, Garcia-Garcia JJ, Pallares R. Emergence of invasive pneumococcal disease caused by non-vaccine serotypes in the era of 7-Valent conjugate vaccine. *Clin Infect Dis* 2008;**46**:174—82.
3. Black S, Shinefield H, Baxter R, Austrian R, Bracken L, Hansen J, et al. Postlicensure surveillance for pneumococcal invasive disease after use of heptavalent pneumococcal conjugate vaccine in northern California Kaiser Permanente. *Pediatr Infect Dis J* 2004;**23**:485—9.
4. Whitney CG, Farley MM, Hadler J, et al. Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* 2003;**348**: 1737—46.
5. Hicks LA, Harrison LH, Flannery B, et al. Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998—2004. *J Infect Dis* 2007;**196**: 1346—54.
6. Singleton RJ, Hennessy TW, Bulkow LR, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* 2007;**297**:1784—92.
7. Beall B, McEllistrem MC, Gertz Jr RE, et al. Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. *J Clin Microbiol* 2006;**44**:999—1017.
8. Park SY, Moore MR, Bruden DL, et al. Impact of conjugate vaccine on Transmission of antimicrobial-resistant *Streptococcus pneumoniae* among Alaskan children. *Pediatr Infect Dis J* 2008;**27**:335—40.
9. Porat N, Arguedas A, Spratt BG, et al. Emergence of penicillin-nonsusceptible *Streptococcus pneumoniae* clones expressing serotypes not present in the antipneumococcal conjugate vaccine. *J Infect Dis* 2004;**190**:2154—61.
10. Sousa NG, Sa-Leao R, Crisostomo MI, Simas C, Nunes S, Frazão N, et al. Properties of novel international drug-resistant pneumococcal clones identified in day-care centers of Lisbon, Portugal. *J Clin Microbiol* 2005;**43**:4696—703.
11. Huang SS, Platt R, Rifas-Shiman SL, Pelton SI, Goldmann D, Finkelstein JA. Post- PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics* 2005;**116**:e408—13.
12. Haber M, Barskey A, Baughman W, Barker L, Whitney CG, Shaw KM, et al. Herd immunity and pneumococcal conjugate vaccine: a quantitative model. *Vaccine* 2007;**25**:5390—8.
13. O'Brien KL, Millar EV, Zell ER, Bronsdon M, Weatherholtz R, Reid R, et al. Effect of pneumococcal conjugate vaccine on nasopharyngeal colonization among immunized and unimmunized children in a community-randomized trial. *J Infect Dis* 2007; **196**:1211—20.
14. Moore MR, Gertz Jr RE, Woodbury RL, Barkocy-Gallagher GA, Schaffner W, Lexau C, et al. Population Snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States. *J Infect Dis* 2008;**197**:1016—27.
15. Pai R, Moore MR, Pilishvili T, Gertz RE, Whitney CG, Beall B. Active bacterial Core surveillance Team. Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. *J Infect Dis* 2005;**192**: 1988—95.
16. Messina AF, Katz-Gaynor K, Barton T, Ahmad N, Ghaffar F, Rasko D, et al. Impact of the pneumococcal conjugate vaccine on serotype distribution and antimicrobial resistance of invasive Streptococcus pneumoniae isolates in Dallas, TX, children from 1999 through 2005. *Pediatr Infect Dis J* 2007;**26**:461—7.
17. Choi EH, Kim SH, Eun BW, Kim SJ, Kim NH, Lee J, et al. *Streptococcus pneumoniae* serotype 19A in children, South Korea. *Emerg Infect Dis* 2008;**14**:275—81.
18. Dagan R, Givon-Lavi N, Leibovitz E, Greenberg D, Porat N. Introduction and Proliferation of multidrug-resistant *Streptococcus pneumoniae* serotype 19A clones that cause Acute Otitis Media in an Unvaccinated population. *J Infect Dis* 2009;**199**:776—85.
19. Pichichero ME, Casey JR. Emergence of a multiresistant serotype 19A pneumococcal strain not included in the 7-valent conjugate vaccine as an otopathogen in children. *JAMA* 2007;**298**:1772—8.
20. Tarrago D, Fenoll A, Sanchez-Tatay D, Arroyo LA, Muñoz-Almagro C, Esteva C, et al. Identification of pneumococcal serotypes from culture-negative clinical specimens by novel Real-Time PCR. *Clin Microbiol Infect* 2008;**14**:828—34.
21. National Committee for Clinical Laboratory Standards (NCCLS). *Performance Standards for antimicrobial susceptibility testing*. Eighteenth informational supplement. CLSI document M100-S18 (ISBN 1-5-56238-653-0). Wayne Pa: Clinical and Laboratory Standard Institute; 2008.
22. Enright MC, Spratt BG. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. *Microbiology* 1998;**144**: 3049—60.
23. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004;**186**:1518—30.
24. American Academy of Pediatrics, Committee on Infectious Diseases. Policy statement: recommendations for the prevention of pneumococcal infections, including the use of pneumococcal conjugate vaccine, and antibiotic prophylaxis. *Pediatrics* 2000; **106**:362—6.
25. Calbo E, Díaz A, Cañadell E, Uriz S, Xercavins M, Morera MA, et al. Invasive pneumococcal disease among children in

a health district of Barcelona: early impact of pneumococcal conjugate vaccine. *Clin Microbiol Infect* 2006;**12**:867—72.
26. Leimkugel J, Adams Forgor A, Gagneux S, Pflüger V, Flierl C, Awine E, et al. An outbreak of serotype 1 Streptococcus pneumoniae meningitis in northern Ghana with features that are characteristic of Neisseria meningitidis meningitis epidemics. *J Infect Dis* 2005;**192**:192—9.
27. Ongkasuwan J, Valdez TA, Hulten KG, Mason Jr EO, Kaplan SL. Pneumococcal mastoiditis in children and the emergence of multidrug-resistant serotype 19A isolates. *Pediatrics* 2008;**122**:34—9.
28. Brueggemann AB, Pai R, Crook DW, Beall B. Vaccine escape recombinants emerge after Pneumococcal vaccination in the United States. *PLoS Pathog* 2007;**3**:e168.
29. Coffey TJ, Dowson CG, Daniels M, Zhou J, Martin C, Spratt BG, et al. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. *Mol Microbiol* 1991;**5**:2255—60.
30. Coffey TJ, Enright MC, Daniels M, Wilkinson P, Berrón S, Fenoll A, et al. Serotype 19A variants of the Spanish serotype 23F multiresistant clone of *Streptococcus pneumoniae*. *Microb Drug Resist* 1998;**4**:51—5.
31. Serrano I, Melo-Cristino J, Carrico JA, Ramirez M. Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal. *J Clin Microbiol* 2005;**43**:1706—15.
32. Farrell DJ, Morrissey I, Bakker S, Morris L, Buckridge S, Felmingham D. Molecular epidemiology of multiresistant *Streptococcus pneumoniae* with both *erm*(B)- and *mef*(A)-mediated macrolide resistance. *J Clin Microbiol* 2004;**42**:764—8.
33. Pelton SI, Huot H, Finkelstein JA, Bishop CJ, Hsu KK, Kellenberg J, et al. Emergence of 19A as virulent and multidrug resistant Pneumococcus in Massachusetts following universal immunization of infants with pneumococcal conjugate vaccine. *Pediatr Infect Dis J* 2007;**26**:468—72.
34. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG. Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis* 2003;**187**:1424—32.
35. Sandgren A, Sjostrom K, Olsson-Liljequist B, Christensson B, Samuelsson A, Kronvall G, et al. Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*. *J Infect Dis* 2004;**189**:785—96.
36. Reinert RR, Jacobs MR, Appelbaum PC, et al. Relationship between the original multiply resistant South African isolates of *Streptococcus pneumoniae* from 1977 to 1978 and contemporary international resistant clones. *J Clin Microbiol* 2005;**43**:6035—41.