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Impact of molecular methods in the analysis of the invasiveness of *Streptococcus pneumoniae*

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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.

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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 56°C followed by a 10-minute incubation at 100°C. 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 900 nM. The TaqMan probes were used at a final concentration of 250 nM. DNA Amplification was done performing universal amplification conditions: incubation for 2 min at 50°C (uracil-N-glycosylase digestion) and 10 min denaturation at 95°C, 45 cycles of 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 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

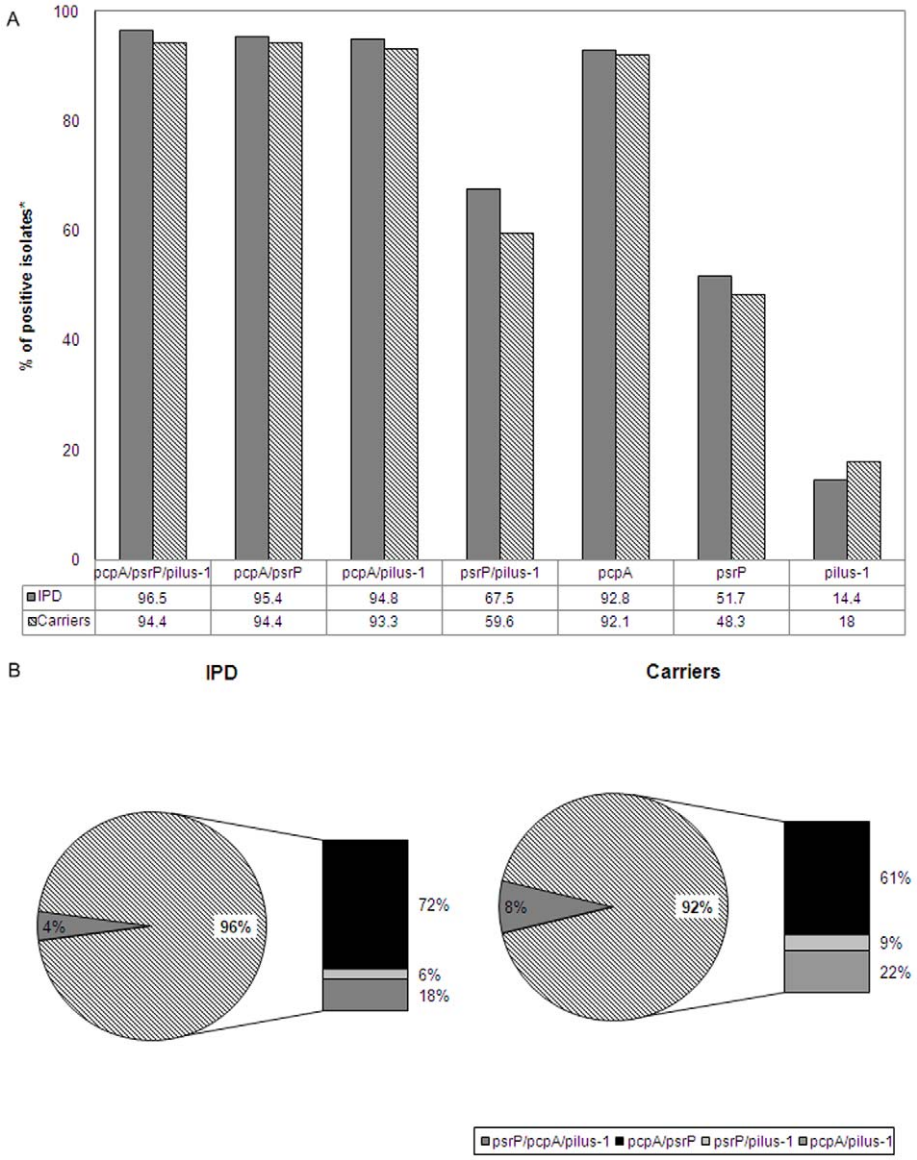

Figure 1. Prevalence of *pcpA, psrP* and pilus-1. (A) Prevalence for *pcpA, psrP* and pilus-1 alone and for their combinations (isolates with al least one of the three combinations) in 458 pneumococcal isolates of patients with invasive pneumococcal disease (IPD) and in 89 pneumococcal isolates of healthy 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.
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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.

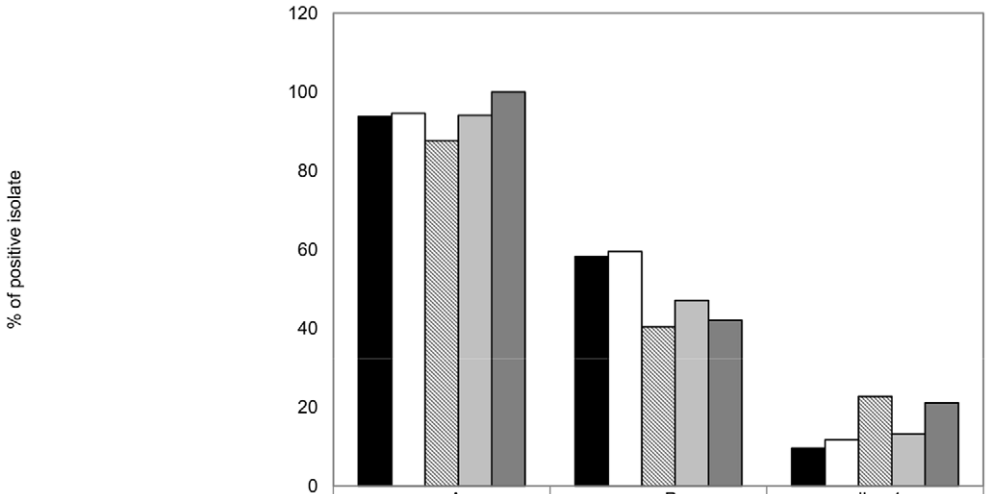
Results

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).



	pcpA	psrP	pilus-1
Non complicated pneumonia (N=146)	93.8	58.2	9.6
Empyema (N=111)	94.6	59.5	11.7
Bacteremia/Sepsis (N=114)	87.7	40.4	22.8
Meningitis (N=68)	94.1	47.1	13.2
Others (N=19)	100	42.1	21.1

Figure 2. Prevalence of *pcpA, psrP* and pilus-1 according to clinical syndrome among pneumococcal invasive isolates.
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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.

Table 1. Prevalence of *pcpA, psrP* and pilus-1 according to antimicrobial susceptibility.

Antimicrobial agent			**pcpA**		**psrP**		**pilus-1**	
MIC	**Isolates**	**%**	**%Positive**	**P**	**%Positive**	**P**	**%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	

The study was non-available in four* and six** isolates.
doi:10.1371/journal.pone.0041587.t001

Table 2. Prevalence of *pcpA,psrP* and Pilus-1 according to serotype of isolates.

Serotype	Isolates	*pcpA* Pos	%	*psrP* Pos	%	pilus Pos	%
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

Pos: positive detection.
doi:10.1371/journal.pone.0041587.t002

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

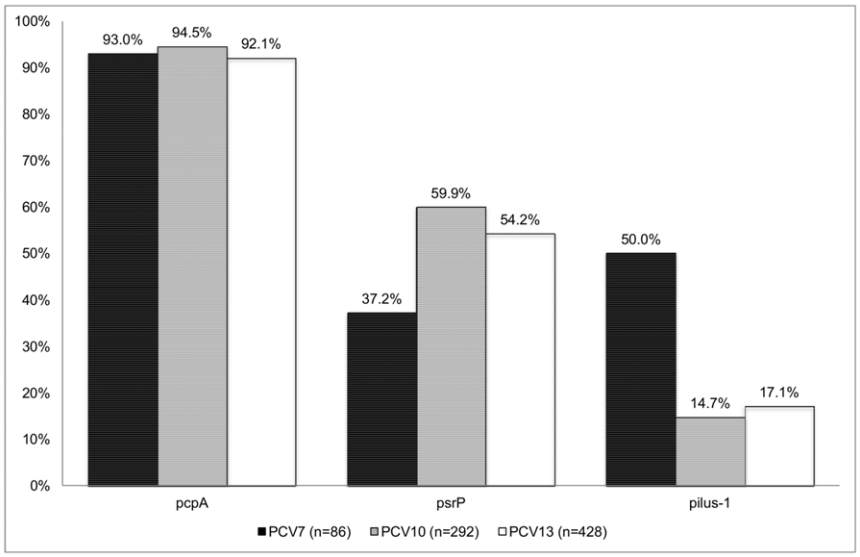

Figure 3. Prevalence of *pcpA, psrP* and pilus-1 according to serotypes within the commercialized conjugate vaccines.
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Table 3. Prevalence of *pcpA. psrP* and pilus-1 according to clonotypes (ST) detected in the study.

			pcpA		psrP		pilus-1	
ST	**Isolates**	**Serotype**	**Positive**	**% Positive**	**Positive**	**% Positive**	**Positive**	**% 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
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

Table 3. Cont.

			pcpA		psrP		pilus-1	
ST	**Isolates**	**Serotype**	**Positive**	**% Positive**	**Positive**	**% Positive**	**Positive**	**% Positive**
		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.

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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 appear highly mucoid on blood agar plates. The absence of these adhesins and a distinct clinical profile suggest that serotype 3 isolates might have a pathogenesis dissimilar to other pneumococcal isolates, as numerous studies indicate that capsular polysaccharide inhibits bacterial adhesion, and serotype 3 isolates are frequently associated with necrotizing pneumonia. This suggests that a distinct protein vaccine formulation would be required for protection against serotype 3-mediated disease. This notion is supported by studies in experimentally infected mice, where a serotype 3 clinical isolate remained in the lungs but replicated to high titers, whereas clinical isolates of serotype 2 and 4 replicated to lower titers but caused disseminated disease [37].

PsrP is both an intraspecies and interspecies adhesin, mediating attachment to Keratin 10 on lung cells and promoting the presence of bacterial aggregates *in vivo* and biofilm formation *in vitro* [38]. Pilus also functions as an adhesin, having been demonstrated to mediate attachment to laminin and may also contribute to the invasiveness of strains [39].

Importantly, considerable evidence indicates that immunization of mice with either the basic region domain of PsrP or with individual components of Pilus-1 mediates protection [16,40]. Using Real-Time PCR, we detected *psrP* in 51.2% of all clinical isolates, whereas we detected pilus-1 in 15% of all isolates. This was consistent with a past study where the prevalence of *psrP* in clinical isolates was found to be 52.4% and with studies of numerous other investigators where the prevalence of pilus-1 in clinical isolates was found to be between 10–30% [30,31,41,42].

Our study expands on these past studies by providing the prevalence of these candidate vaccine antigens simultaneously. There by assessing the potential coverage of a multivalent vaccine composed of *pcpA*, *psrP* and pilus-1. In all, 96% of the strains examined carried at least 1 of these proteins, 96% carried 2, and 6% carried all 3. Our analysis determined that *psrP* and pilus-1 have a negative correlation in multiple serotypes raising the possibility that *psrP* and pilus-1 may have redundant roles, or that their production might be metabolically expensive and that an individual strain cannot support production of both of these extremely large proteins. Briefly, PsrP is a 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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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: RP CJO CMA LS. Revised the paper: all authors.

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Rapid and Easy Identification of Capsular Serotypes of *Streptococcus pneumoniae* by Use of Fragment Analysis by Automated Fluorescence-Based Capillary Electrophoresis

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The purpose of this study was to develop a high-throughput method for the identification of pneumococcal capsular types. Multiplex PCR combined with fragment analysis and automated fluorescent capillary electrophoresis (FAF-mPCR) was utilized. FAF-mPCR was composed of only 3 PCRs for the specific detection of serotypes 1, 2, 3, 4, 5, 6A/6B, 6C, 7F/7A, 7C/(7B/40), 8, 9V/9A, 9N/9L, 10A, 10F/(10C/33C), 11A/11D/11F, 12F/(12A/44/46), 13, 14, 15A/15F, 15B/15C, 16F, 17F, 18/(18A/18B/18C/18F), 19A, 19F, 20, 21, 22F/22A, 23A, 23B, 23F, 24/(24A/24B/24F), 31, 33F/(33A/37), 34, 35A/(35C/42), 35B, 35F/47F, 38/25F, and 39. In order to evaluate the assay, all invasive pneumococcal isolates ($n = 394$) characterized at Hospital Sant Joan de Déu, Barcelona, Spain, from July 2010 to July 2011 were included in this study. The Wallace coefficient was used to evaluate the overall agreement between two typing methods (Quellung reaction versus FAF-mPCR). A high concordance with Quellung was found: 97.2% (383/394) of samples. The Wallace coefficient was 0.981 (range, 0.965 to 0.997). Only 11 results were discordant with the Quellung reaction. However, latex reaction and Quellung results of the second reference laboratory agreed with FAF-mPCR for 9 of these 11 strains (82%). Therefore, we considered that only 2 of 394 strains (0.5%) were not properly characterized by the new assay. The automation of the process allowed the typing of 30 isolates in a few hours with a lower cost than that of the Quellung reaction. These results indicate that FAF-mPCR is a good method to determine the capsular serotype of *Streptococcus pneumoniae*.

Pneumococcal disease is a bacterial infection caused by *Streptococcus pneumoniae*. Its morbidity and mortality have a huge impact on global health. The organism causes at least 1.6 million deaths each year worldwide, of which 800,000 occur among children aged under 5 years (19).

The capsular polysaccharide represents an important virulence factor and characterizes *S. pneumoniae* by 93 distinct serotypes (8). Only certain polysaccharide types appear to be more liable to cause invasive disease (7, 16). Conjugate vaccines against serotypes 7, 10, and 13 (PCV7, PCV10, and PCV13) have been shown to be effective in preventing pneumococcal infections (32, 35, 38). However, the increase of disease caused by nonvaccine serotypes is a special concern (21, 42). Therefore, continuous monitoring of changes in serotype distribution is necessary for epidemiological surveillance and evaluation of vaccines.

The Quellung reaction or Neufeld test is the gold standard for serotyping (2). This test requires isolation of pneumococci by culture followed by serological determination of the capsular type by using specific polyclonal antibodies. The antibody binds the pneumococcal capsule and induces a capsular swelling that is visible under the microscope. Quellung reaction is expensive because it is necessary to buy all antisera to identify the different serotypes. In addition, it is labor-intensive because pneumococcal isolates are sequentially tested first against pooled antisera and then against each of the individual antisera included in the reactive pool. Subjective interpretation and technical expertise requirements are other serious drawbacks of the system.

Other methods used to perform pneumococcal serotyping are dot blot assay (15) and latex agglutination test (36). The interpretation of blots is time-consuming and is not recommended for routine serotyping (6). The latex agglutination test is an easy and rapid test but is also expensive and limited to a few serogroups/serotypes. Several authors have published multiplex PCR schemes to deduce specific pneumococcal serotypes from isolate sets (20, 29) and also from clinical specimens (3, 23, 31, 33, 44). Multiplex PCR-based methods offer a simple and economical approach for the surveillance of pneumococcal disease. The main advantages in relation to classical serotyping are a lower cost than Quellung reaction (29), rapidity (39), and the capacity to detect secondary serotypes in mixed cultures (29). However, these developed schemes are limited to a small number of primer pairs per reaction (from two to four primers pairs); therefore, it is necessary to perform up to eight sequential multiplex reactions to identify 30 different serotypes/serogroups (44). Other molecular technologies for rapid *S. pneumoniae* serotyping have been described. Elberse et al. (14) have developed the capsular sequence typing (CST) method to genotype the capsular locus in order to assess the serotype. Bentley et al. (4) have designed a microarray on the basis of serotype-specific oligonucleotides within the *cps* locus for 90 known serotypes. Recently, Yu et al. (43) have developed a pneumococcal serotyping system which combines the PCR and monoclonal antibody (MAb)-based approaches that can test all known serotypes.

Capillary electrophoresis by using multicapillary automated DNA sequencers has been widely used in the separation of PCR products. It offers advantages of fast separation, high-resolution analysis, and small reagent consumption over traditional agarose

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gel electrophoresis (17, 22, 41). The fluorescent PCR-based technology combined with fragment analysis by automated fluorescent capillary electrophoresis could greatly simplify the serotyping of pneumococci with good sensitivity and specificity. The assay combines the power of multiplex PCR with the use of fluorescently labeled primer pairs and provides high detection sensitivity of amplified fragments (22). This technology is being applied successfully for the genotyping of human papillomavirus (9, 24, 40) and can be developed with the simplicity expected for the characterization of *S. pneumoniae*.

The purpose of this study was to develop a method for the identification of pneumococcal capsular types by multiplex PCR combined with fragment analysis and automated fluorescent capillary electrophoresis (FAF-mPCR) and to compare it to conventional serotyping.

MATERIALS AND METHODS

Bacterial isolates. In order to evaluate the assay, all invasive pneumococcal isolates characterized by the Molecular Microbiology Department at University Hospital Sant Joan de Déu in Barcelona, Spain, from July 2010 to July 2011 were included in this study. The department performs molecular surveillance of pneumococci from patients attending 30 health centers throughout the Catalonia region. These 30 health centers captured 40.5% of all hospital admissions and, specifically, 63% of pediatric hospital admissions during 2009 (27).

Isolates were cultured on blood agar plates (Columbia agar supplemented with 5% sheep blood; bioMérieux) and were incubated overnight at 37°C in 5% CO_2. Each pneumococcal isolate was identified using standard microbiological tests, including colony morphology and optochin susceptibility.

***S. pneumoniae* serotyping by Quellung reaction.** All isolates were serotyped by Quellung reaction at the National Pneumococcus Reference Centre (Majadahonda, Madrid, Spain) by using rabbit polyclonal antiserum from the Statens Serum Institute (Copenhagen, Denmark) and the chessboard system as previously described (37). Briefly, the Quellung reaction was performed by adding a drop of polyvalent *S. pneumoniae* antiserum to an air-dried drop of the pneumococcal isolate to be studied. A reaction to the antiserum being tested was considered positive when capsular swelling was observed with a phase-contrast microscope (magnification, ×1,000). The capsular test was carried out with 14 successively antiserums pools and 46 specific antiserum groups or types until a positive reaction was obtained.

Pneumococcal isolates with serotype discordance between Quellung and FAF-mPCR were serotyped in our laboratory by latex agglutinations using the Pneumotest latex kit (Statens Serum Institut, Copenhagen, Denmark). In addition, these strains were also sent to another international reference laboratory to be retyped by Quellung reaction.

Molecular serotyping of *S. pneumoniae*. (i) DNA extraction from bacterial isolates. Genomic DNA was extracted from bacteria using Chelex-100 resin (Bio-Rad Laboratories). Briefly, pneumococci scraped from blood agar plates were 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 bacterial/resin suspensions were incubated for 20 min at 56°C, followed by a 10-min incubation at 100°C. After cooling and centrifugation, the supernatant was used as a DNA template in PCRs.

(ii) Multiplex PCR combined with fragment analysis detection by automated fluorescent capillary electrophoresis. Forty primer pairs were used to target serotypes 1, 2, 3, 4, 5, 6A/6B, 6C, 7F/7A, 7C/(7B/40), 8, 9V/9A, 9N/9L, 10A, 10F/(10C/33C), 11A/11D/11F, 12F/(12A/44/46), 13, 14, 15A/15F, 15B/15C, 16F, 17F, 18/(18A/18B/18C/18F), 19A, 19F, 20, 21, 22F/22A, 23A, 23B, 23F, 24/(24A/24B/24F), 31, 33F/(33A/37), 34, 35A/(35C/42), 35B, 35F/47F, 38/25F, and 39 (Table 1). Some serotypes were indistinguishable from other closely related serotypes, most of which belonged to the same serogroup. The sequences for the type-specific primers have been published by different authors (10, 12, 29, 30) and are available at the CDC web page (http://www.cdc.gov/ncidod/biotech/strep/pcr.htm). Primers were previously designed on the basis of DNA sequences of the *wzy* gene [serotypes 1, 2, 4, 5, 7F/A, 8, 9V/A, 11A/D/F, 14, 15A/F, 15B/C, 16F, 18/(18A/18B/18C/18F), 19A, 19F, 23A, 23F, 24/(24A/24B/24F), 31, 33F/(33A/37), 34, 35F/47F, 38/25F, 39] and *wzx* gene [serotype 9N/9L, 10F/(10C/33C), 12F/(12A/44/46), 13, 21, 23B, 35A/(35C/42)], except for serotypes 3 (*galU*), 6A/6B and 17F (*wciP*), 7C/(7B/40) (*wcwL*), 10A (*wcrG*), 20 (*wciL*), 22F/A (*wcwV*), and 35B (*wcrH*). Primers specific to the *cpsA* (*wzg*) gene were used as an internal positive control. This gene is part of the *cps* locus and is very common to all capsular serotypes.

In the present study, forward primers of each pair of primers were labeled with 6-carboxyfluorescein (FAM), 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC), 2′-chloro-5′-fluoro-7′,8′-benzo-1,4-dichloro-6-carboxyfluorescein (NED), or PET fluorochrome; primers pairs were synthesized by Applied Biosystems. In addition, primers were grouped together based on the size of the fragment and the fluorochrome selected.

The PCRs were carried out in a final volume of 25 μl with 12.5 μl of 2× Qiagen multiple PCR master mix (Qiagen), primers at the concentrations specified in Table 2, and distilled water to a final volume of 23 μl. Two microliters of DNA extract were used as the template for each PCR. Thermal cycling was performed in a GeneAmp PCR system 9700 (Applied Biosystems) under the following conditions: 95°C for 15 min, followed by 25 amplification cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 60 s, and a final extension step at 72°C for 10 min. Each amplification run contained a negative control (water).

Fluorescent fragment size analysis was performed on an ABI 3130xl genetic analyzer (Applied Biosystems). Data were collected with ABI 3130xl data collection software (version 3.0; Applied Biosystems) and interpreted using GeneMapper software (version 4.0; Applied Biosystems). For capillary electrophoresis, 2-μl aliquots were added to the capillary electrophoresis mixture containing 20 μl of Hi-Di formamide (Applied Biosystems) and 0.6 μl of the GeneScan 1200 LIZ size standard (Applied Biosystems). The capillary sample mixture was denatured for 5 min at 95°C and rapidly cooled on ice prior to analysis. The ABI 3130xl genetic analyzer was set up according to the manufacturer's instructions to use performance-optimized POP-7 polymer for microsatellite analysis.

Economic evaluation of FAF-mPCR versus Quellung reaction. Economic cost of reagents and manual labor for FAF-mPCR were compared to the chessboard typing system of the Quellung reaction. We considered the cost of all reagents and hands-on labor time necessary to achieve a serotype result by Quellung versus FAF-mPCR.

Statistical analysis. The congruence between Quellung reaction and the new method was studied by means of the Wallace coefficient (34). This coefficient indicates the probability that a pair of isolates which is assigned to the same type by one typing method is also typed as identical by another method (13). All calculations were performed using the freely available online tool Comparing Partitions, located at www.comparingpartitions.info.

RESULTS

During the study period, a total of 394 invasive isolates were received in our laboratory and 37 serogroup/serotypes were assigned by Quellung reaction. Nine serotypes (7C, 7B, 10F, 10C, 33C, 35A, 35C, 40, and 42) were not available in this collection and could not be included in the comparative analysis with FAF-mPCR. The serotypes/serogroups included in this validation were 1 ($n = 55$), 19A ($n = 40$), 3 ($n = 37$), 7F ($n = 36$), 12F ($n = 26$), 14 ($n = 26$), 22F ($n = 14$), 8 ($n = 11$), 24F ($n = 11$), 5 ($n = 9$), 6C ($n = 9$), 4 ($n = 8$), 9N ($n = 8$), 23F ($n = 8$), 6B ($n = 7$), 9V ($n = 7$), 16F ($n = 7$), 31 ($n = 7$), 10A ($n = 6$), 23A ($n = 6$), 6A ($n = 5$),

TABLE 1 Oligonucleotide primers for each serotype used in the present study

Primer name (forward/reverse)	GenBank accession no.	Forward/reverse primer sequence[a] (5′–3′)	Gene	Product size (bp)
1-f/1-r	CR931632	PET-CTCTATAGAATGGAGTATATAAACTATGGTTA/CCAAAGAAAATACTAACATTATCACAATATTGGC	*wzy*	280
2-f/2-r	CR931633	PET-TATCCCAGTTCAATATTTCTCCACTACACC/ACACAAAATATAGGCAGAGAGAGACTACT	*wzy*	290
3-f/3-r	CR931634	PET-ATGGTGTGATTTCTCCTAGATTGGAAAGTAG/CTTCTCCAATTGCTTACCAAGTGCAATAACG	*galU*	371
4-f/4-r	CR931635	PET-CTGTTACTTGTTCTGGACTCTCGATAATTGG/GCCCACTCCTGTTAAAATCCTACCCGCATTG	*wzy*	430
5-f/5-r	CR931637	NED-ATACCTACACAACTTCTGATTATGCCTTTGTG/GCTCGATAAACATAATCAATATTTGAAAAAGTATG	*wzy*	362
6A/B/C-f/6A/B/C-r	CR931639	FAM-AATTTGTATTTTATTCATGCCTATATCTGG/TTAGCGGAGATAATTTAAAATGATGACTA	*wciP*	250
6C-f/6C-r	EU714777.1	NED-CATTTTAGTGAAGTTGGCGGTGGAGTT/AGCTTCGAAGCCCATACTCTTCAATTA	*wciN* beta	727
7C/(7B/40)-f/7C/(7B/40)-r	CR931642	VIC-CTATCTCAGTCATCTATTGTTAAAGTTTACGACGGGA/GAACATAGATGTTGAGACATCTTTTGTAATTTC	*wcwL*	260
7F/A-f/7F/A-r	CR931643	NED-TCCAAACTATTACAGTGGGAATTACGG/ATAGGAATTGAGATTGCCAAAGCGAC	*wzy*	599
8-f/8-r	CR931644	NED-GAAGAAACGAAACTGTCAGAGCATTTACAT/CTATAGATACTAGTAGAGCTGTTCTAGTCT	*wzy*	201
9N/L-f/9N/L-r	CR931647	FAM-GAACTGAATAAGTCAGATTTAATCAGC/ACCAAGATCTGACGGGCTAATCAAT	*wzx*	516
9V/A-f/9V/A-r	CR931648	VIC-GGGTTCAAAGTCAGACAGTGAATCTTAA/CCATGAATGAAATCAACATTGTCAGTAGC	*wzy*	816
10A-f/10A-r	CR931649	PET-GGTGTAGATTTACCATTAGTGTCGGCAGAC/GAATTTCTTCTTTAAGATTCGGATATTTCTC	*wcrG*	628
10F/(C/33C)-f/10F/(C/33C)-r	CR931652	NED-GGAGTTTATCGGTAGTGCTCATTTTAGCA/CTAACAAATTCGCAACACGAGGCAACA	*wzx*	248
11A/D-f/11A/D-r/	CR931653	FAM-GGACATGTTCAGGTGATTTCCCAATATAGTG/GATTATGAGTGTAATTTATTCCAACTTCTCCC	*wzy*	463
12F/(A/44/46)-f/12F/(A/44/46)-r	CR931660	PET-GCAACAAACGGCGTGAAAGTAGTTG/CAAGATGAATATCACTACCAATAACAAAAC	*wzx*	376
13-f/13-r	CR931661	FAM-TACTAAGGTAATCTCTGGAAATCGAAAGG/TACTAAGGTAATCTCTGGAAATCGAAAGG	*wzx*	655
14-f/14-r	CR931662	VIC-GAAATGTTACTTGGCGCAGGTGTCAGAATT/GCCAATACTTCTTAGTCTCTCAGATGAAT	*wzy*	189
15A/F-f/15A/F-r	CR931663	NED-ATTAGTACAGCTGCTGGAATATCTCTTC/GATCTAGTGAACGTACTATTCCAAAC	*wzy*	434
15B/C-f/15B/C-r	CR931665	PET-TTGGAATTTTTTAATTAGTGGCTTACCTA/CATCCGCTTATTAATTGAAGTAATCTGAACC	*wzy*	496
16F-f/16F-r	CR931668	FAM-GAATTTTTCAGGCGTGGGTG TAAAAG/CAGCATATAGCACCGCTAAGCAAATA	*wzy*	717
17F-f/17F-r	CR931670	FAM-TTCGTGATGATAATTCCAATGATCAAACAAGAG/GATGTAACAAATTTGTAGCGACTAAGGTCTGC	*wciP*	693
18/(A/B/C/F)-f/18/(A/B/C/F)-r	CR931673	NED-CTTAATAGCTCTCATTATTCTTTTTTTAAGCC/ TTATCTGTAAACCATATCAGCATCTGAAAC	*wzy*	573
19A-f/19A-r	CR931675	VIC-GAGAGATTCATAATCTTGCACTTAGCCA/CATAATAGCTACAAATGACTCATCGCC	*wzy*	566
19F-f/19F-r	CR931678	NED-GTTAAGATTGCTGATCGATTAATTGATATCC/GTAATATGTCTTTAGGGCGTTTATGGCGATAG	*wzy*	304
20-f/20-r	CR931679	VIC-GAGCAAGAGTTTTTCACCTGACAGCGAGAAG/CTAAATTCCTGTAATTTAGCTAAAACTCTTATC	*wciL*	514
21-f/21-r	CR931680	VIC-CTATGGTTATTTCAACTCAATCGTCACC/GGCAAACTCAGACATAGTATAGCATAG	*wzx*	192
22F/A-f/22F/A-r	CR931682	PET-GAGTATAGCCAGATTATGGCAGTTTTATTGTC/ CTC CAGCACTTGCGCTGGAAACAACAGACAAC	*wcwV*	643
23A-f/23A-r	CR931683	VIC-TATTCTAGCAAGTGACGAAGATGCG/CCAACATGCTTAAAAACGCTGCTTTAC	*wzy*	722
23B-f/23B-r	CR931684	VIC-CCACAATTAGCGCTATATTCATTCAATCG/GTCCACGCTGAATAAAATGAAGCTCCG	*wzx*	199
23F-f/23F-r	CR931685	VIC-GTA ACA GTT GCT GTA GAG GGA ATT GGC TTT TC/ CAC AAC ACC TAA CAC TCG ATG GCT ATA TGA TTC	*wzy*	384
24/(A,B,F)-f/24/(A,B,F)-r	CR931688	PET-GCT CCC TGC TAT TGT AAT CTT TAA AGA G/ GTGTCT TTT ATT GAC TTT ATC ATA GGT CGG	*wzy*	99
31-f/31-r	CR931695	VIC-GGA AGT TTT CAA GGA TAT GAT AGT GGT GGT GC/ CCG AAT AAT ATA TTC AAT ATA TTC CTA CTC	*wzy*	701
33F/(A/37)-f/33F/(A/37)-r	CR931702	FAM-GAA GGC AAT CAA TGT GAT TGT GTC GCG/CTT CAA AAT GAA GAT TAT AGT ACC CTT CTA C	*wzy*	338
34-f/34-r	CR931703	FAM-GCT TTT GTA AGA GGA GAT TAT TTT CAC CCA AC/ CAA TCC GAC TAA GTC TTC AGT AAA AAA CTT TAC	*wzy*	408
35A/(C/42)-f/35A/(C/42)-r	CR931704	NED-ATT ACG ACT CCT TAT GTG ACG CGC ATA/CCA ATC CCA AGA TAT ATG CAA CTA GGT T	*wzx*	280
35B-f/35B-r	CR931705	PET-GAT AAG TCT GTT GTG GAG ACT TAA AAA GAA TG/ CTT TCC AGA TAA TTA CAG GTA TTC CTG AAG CAA G	*werH*	677
35F/47F-f/35F/47F-r	CR931707	VIC-GAA CAT AGT CGC TAT TGT ATT TTA TTT AAA GCAA/GAC TAG GAG CAT TAT TCC TAG AGC GAG TAA ACC	*wzy*	517
38/25F-f/38/25F-r	CR931710	NED-CGT TCT TTT ATC TCA CTG TAT AGT ATC TTT ATG/ATG TTT GAA TTA AAG CTA ACG TAA CAA TCC	*wzy*	574
39-f/39-r	CR931711	NED-TCA TTG TAT TAA CCC TAT GCT TTA TTG GTG/GAG TAT CTC CAT TGT ATT GAA ATC TAC CAA	*wzy*	98
cpsA-f/*cpsA*-r	CR931662	FAM-GCA GTA CAG CAG TTT GTT GGA CTG ACC/GAA TAT TTT CAT TAT CAG TCC CAG TC	*wzg*	160

[a] Forward primers labeled with FAM, VIC, NED or PET fluorochromes.

TABLE 2 Primer concentrations in the three multiplex PCRs used in the present study

Reaction and primers	Primer concn (μM)
1	
8-f, 8-r, 39-f, 39-r	0.1
14-f, 14-r, 1-f, 1-r, 33F/(33A/37)-f, 33F/(33A/37)-r, 15A/15F-f, 15A/15F-r, 15B/15C-f	0.15
15B/15C-r, 35F/47F-f, 35F/47F-r, 6A/6B-f	0.15
6A/6B-r, 7C/7B/40-f, 7C/7B/40-r, 5-f, 5-r, 3-f, 3-r	0.15
23F-f, 23F-r	0.3
2	
21-f, 21-r	0.1
35A/(35C/42)-f, 35A/(35C/42)-r, 11A/11D/11F-f, 11A/11D/11F-r, 4-f, 4-r, 19A-f, 19A-r	0.15
7F/7A-f, 7F/7A-r, 16F-f, 16F-r	0.2
12F/(12A/44/46)-f, 12F/(12A/44/46)-r, 22F/22A-f, 22F/22A-r	0.3
17F-f, 17F-r, 23A-f, 23A-r, 19F-f, 19F-r, 35B-f, 35B-r, 9V/9A-f	0.3
9V/9A-r, 20-f, 20-r	0.3
3	
24/(24,24B,24F)-f, 24/(24,24B,24F)-r, 23B-f, 23B-r	0.1
10F/(10C/33C)-f, 10F/(10C/33C)-r, 2-f, 2-r, 34-f, 34-r, 38/25F-f	0.15
38/25F-r, 18/(18A/18B/18C/18F)-f, 18/(18A/18B/18C/18F)-r	0.15
9N/L-f, 9N/L-r, 10A-f, 10A-r, 13-f, 13-r, 31-f, 31-r, 6C-f, 6C-r	0.2

11A ($n = 5$), 19F ($n = 5$), 23B ($n = 5$), 38 ($n = 5$), 11F ($n = 4$), 15B ($n = 4$), 15A ($n = 3$), 17F ($n = 3$), 18C ($n = 3$), 15C ($n = 2$), 29 ($n = 2$), 33F ($n = 2$), 34 ($n = 2$), 35B ($n = 2$), 20 ($n = 1$), 35F ($n = 1$), 39 ($n = 1$), and nontypeable ($n = 1$). Therefore, 62.4% of them were serotypes included in PCV13, 41.6% were included in PCV10, and 16.2% were in PCV7.

Selection of primers and optimization of three multiplex PCRs. The primers were ordered according to the different sizes of products (range, 98 to 816 bp). Distinct fluorophores were used in the products with the most similar sizes in order to obtain better discrimination of products.

The concentrations of the primers were adjusted to achieve similar levels of amplification of the products with the same amplification conditions and to avoid unspecific products in the reaction. Finally, we combined all primers in three multiplex PCRs as shown in Table 2. All three reactions included primers to detect *cpsA* as an internal control.

Concordance of serotyping by FAF-mPCR versus Quellung reaction. Results of the FAF-mPCR and Quellung reaction agreed for 97.2% (383/394) of the samples. Specific detection of serotype by FAF-mPCR was accurate and objective, as shown in Fig. 1. The congruence between FAF-mPCR and Quellung reaction as examined by the Wallace coefficient was 0.981 (range, 0.965 to 0.997). Only 11 results were discordant with the Quellung reaction. However, latex and Quellung reaction results of the second reference laboratory agreed with FAF-mPCR for 9 of the 11 (82%) strains (Table 3). Therefore, we considered that only 2 of 394 strains (0.5%) were not properly characterized by the new assay.

Comparison of economic cost of FAF-mPCR to that of Quellung reaction. In the present study, we performed the three reactions with all isolates in order to validate the technique, but we considered a sequential approach to the three reactions with the purpose of estimating the cost of the new technique. Among the 394 isolates, serotypes of 171 (43.4%) isolates were detected in the first reaction with a reagent cost of €605, 164 (41.6%) were detected in the second reaction (estimated cost of €1,331), and 56 (14.2%) were detected in the third reaction (estimated cost of €1,499). Finally, 3 (0.7%) isolates were not detected in any reaction (estimated cost of €32.9). Two of these isolates corresponded to serotype 29 by Quellung reaction, which is not included in the typing scheme of FAF-mPCR, and the third isolate corresponded to a nontypeable serotype by either of the two techniques. Therefore, the total PCRs performed for serotyping the 394 isolates were 676 with a total cost of €2,560 (€6.5 per isolate), while a total of 2,924 reactions were needed to complete the serotyping of isolates by Quellung reaction, with a total cost of €6,433 (€16.3 per isolate).

The FAF-mPCR has a high throughput, and a batch of 90 reactions can be carried out in 90 min. Consequently, the 676 reactions were performed in 11.3 h of manual labor. In contrast, a minimum of 3 min is necessary for each Quellung reaction. Therefore, the 2,924 reactions performed according to the chessboard system of Quellung needed a total of 146.2 h of work.

DISCUSSION

To our knowledge, this is the first report that determines the capsular type of *S. pneumoniae* using fragment analysis by automated fluorescent capillary electrophoresis. The assay described in this article assigned the serotype of 99.2% of our isolates by using only three PCRs. Moreover, two of the three isolates not characterized by the new assay were not characterized by Quellung either (nontypeable isolates). This multiplex PCR is rapid and easy and only needs basic PCR knowledge that is already broadly available in microbiology departments.

The concordance of results with Quellung reaction was high; moreover, the few discrepancies between Quellung and FAF-mPCR were resolved in favor of FAF-mPCR when we used latex agglutination and when samples were retyped by Quellung reaction in another laboratory. The reading of results of FAF-mPCR is objective and does not demand a high level of expertise for the interpreta-

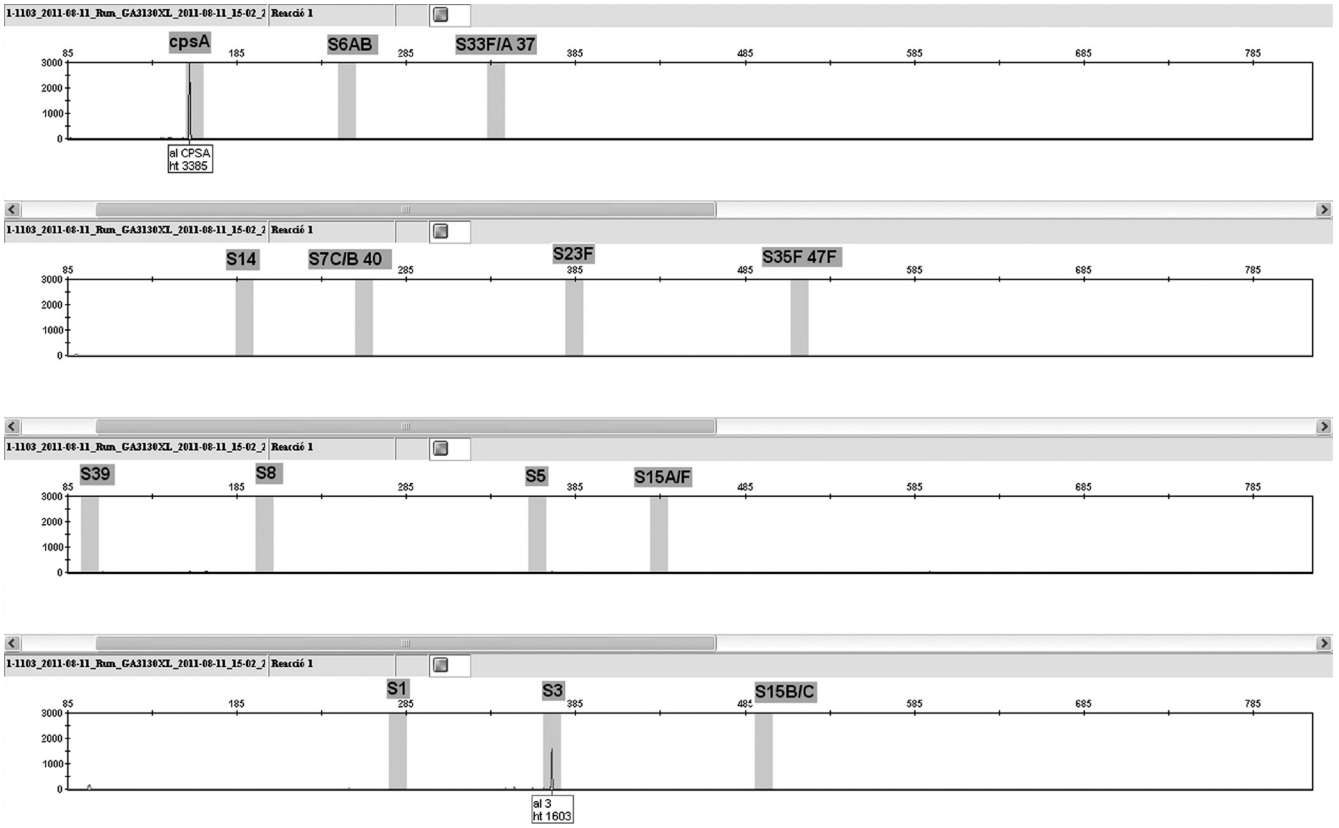

FIG 1 Electropherogram of reaction 1 with the positive detection of serotype 3.

tion of results. In contrast, Quellung reaction is subjective and a high level of experience is required to assign the correct serotype.

Different authors have adapted the original multiplex PCR assay developed by Pai et al. (29) and recommended by the CDC (1, 3, 11, 12, 18, 26, 28, 31). All authors agree that multiplex PCR is an accurate, simple, and economical method which can be used for determining capsular type. However, the classical multiplex PCR approach used by the different authors implies a high number of PCRs. Due to technical limitations, only a small number of primers (maximum of 4) can be used in each reaction. The use of multiple assay tubes to prepare the different PCRs increases the risk for pipetting errors and contaminations. The FAF-mPCR method improves on the original multiplex PCR by using fragment analysis by automated fluorescent capillary electrophoresis. This technology becomes a fast, easy, and accurate system for PCR product detection and interpretation of results. The utilization of primers labeled with fluorescent dyes allows multiplexing more than 15 sets of primers in a single PCR mixture. After PCR amplification, PCR products of similar size but marked with different fluorophors can be distinguished from one another without overlap by using automating software. The system recognizes fluorescent peaks according to specific color and size and awards the corresponding capsular type accurately. In addition, the use of automatic DNA sequencers enables a high-throughput analysis, and a single technician can process a large number of samples in a few hours.

Our system has some limitations. Like in other multiplex PCRs, we cannot identify some serotypes (3, 25, 26, 44) or distinguish genetically related serotypes (i.e., 7C/7B/40 and 10F/10C/33C). In order to identify these serotypes, it is still necessary to perform conventional manual methods based on polyclonal factor sera or specific DNA tests.

The present assay was validated in clinical isolates from patients with invasive pneumococcal disease. The next step will be to validate assay performance directly on clinical samples from culture-negative patients.

TABLE 3 Analysis of discrepant results[a]

Strain	Result by: FAF-mPCR	Quellung 1	Quellung 2	Latex agglutination
1	18	22F	18B	18(18F/18A/18B/18C)
2	11A/11D	31	11A	11(11F/11A/11B/11C/11D)
3	10A	11F	10A	10(10F/10A/10B/10C)
4	7F/A	31	7A	7(7F/7A/7B/7C)
5	22F/A	7F	22F	22(22F/22A)
6	3	18C	3	3
7	7F/A	31	7A	7(7F/7A/7B/7C)
8	5	17F	5	5
9	19A	23F	19A	19(19F/19A/19B/19C)
10	18	38	25A	25(25F/25A), 38, 43, 44, 45, 46, 48
11	18	38	25A	25(25F/25A), 38, 43, 44, 45, 46, 48

[a] FAF-mPCR, multiplex PCR combined with fragment analysis and automated fluorescent capillary electrophoresis; Quellung 2, results of retyped strains by Quellung reaction.

According to other authors (5, 29), compared to Quellung reaction, multiplex PCR systems are cost-effective in terms of reagent costs and labor time requirements. This is also valid for FAF-mPCR, as shown in our cost analysis. However, we must consider that FAF-mPCR requires expensive instrumentation (automatic DNA sequencer). In spite of this, multicapillary automated DNA sequencers are highly available in the clinical laboratories because these instruments are utilized in numerous laboratory tasks. Therefore, the requirement of sequencer use would not be an important limitation for the introduction of the new technique.

In conclusion, the FAF-mPCR method for identification of capsular serotypes of *S. pneumoniae* may be a useful alternative to more widely accepted methodologies, particularly multiplex PCR.

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Pneumococcal carriage in children attending a hospital outpatient clinic in the era of pneumococcal conjugate vaccines in Barcelona ☆

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ABSTRACT

Between April 2004 and March 2006 an oropharyngeal swab was obtained from 502 asymptomatic children, aged 6 months to 6 years, at a tertiary children's hospital outpatient department to assess the pneumococcal colonisation rate, risk factors, serotype distribution and antimicrobial susceptibility. Only 126 (25.3%) children had received ≥ 1 dose of PCV7. The pneumococcal carriage rate was 23.5%. Carrier rates were significantly higher in children aged ≥ 24 months and children attending daycare center. Thirty six (31.0%) of the isolates were contained in PCV7, 39 (33.6%) in PCV10 and 62 (53.4%) in PCV13. Forty-four strains (37.9%) were resistant to penicillin. Vaccine serotype (VT) strains were more likely to be penicillin-nonsusceptible *S. pneumoniae* than non-PCV7 serotype (NVT) strains (66.7% vs. 21.6%; $P < 0.001$). In our pediatric population, NVT were predominant among pneumococcal carriers whereas antibiotic resistance was significantly associated with VT. PCV13 can substantially increase the serotype coverage of *S.pneumoniae* in healthy carriers.

1. Introduction

Streptococcus pneumoniae is a leading cause of invasive disease, such as meningitis and bacteraemia, and the most frequent bacterial cause of community-acquired pneumonia, otitis media and sinusitis in paediatric populations worldwide. Children aged less than two years are at greatest risk.

Pneumococcus commonly colonizes the upper respiratory tract of healthy children and is easily transmitted, usually by droplet secretions, from person to person. When the balance between host and pathogen is disturbed, *S. pneumoniae* can spread to adjacent mucosal tissues to cause mucosal infections or invade the blood-stream to cause invasive infections. Although most children are colonised at some point during the first two years of life, only a small minority will develop an invasive infection (Bogaert et al., 2004).

Several factors have been associated with increased prevalence of carriage, including overcrowding (e.g., attending daycare centres (DCC), residing in orphanages), younger age, family contacts, exposure to cigarette smoke, colder months of the year, frequent respiratory tract infections and an excessive use of antibiotics (Principi et al., 1999).

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the United States in 2000 was followed by a significant reduction in invasive pneumoccocal disease (IPD) and nasopharyngeal (NP) carriage due to vaccine serotype (VT) rates (Black et al., 2000; Whitney et al., 2003). Despite the effectiveness of this vaccine, the emergences of nonvaccine serotypes (NVT) in both IPD and NP isolates and changes in antimicrobial susceptibilities have been documented around the world [Frazao et al., 2005; Gonzalez et al., 2006; Farrell et al., 2007; Muñoz-Almagro et al., 2008; Muñoz-Almagro et al., 2009; Sa-Leao et al., 2009; Huang et al., 2009). In 2010, two pneumococcal conjugate vaccines that included additional emerging serotypes were licensed: the 10-valent pneumococcal conjugate vaccine (PCV10; Synflorix®), adding serotypes 1, 5, and 7F, and the 13-valent pneumococcal conjugate vaccine (PCV13; Prevenar-13®), adding serotypes 1, 3, 5, 6A, 7F, and 19A.

PCV7 became available in Spain in June 2001, although at present in our region (Catalonia) it is not subsidized by the national public health system. A study carried out in 2005 in Catalonia found an estimated vaccination rate of 30% (Calbo et al., 2006). There are limited data on pneumococcal serotypes involved in carriage in our country, particularly since the introduction of PCV7 (García de Lomas et al., 1997; Lopez et al., 1999; Sánchez-Tatay et al., 2008).

Because NP carriage is a major factor in the transmission of pneumoccocal disease, continuing careful surveillance of colonized

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children is essential to increase understanding of changes in the serotype distribution and antibiotic susceptibility of IPD isolates.

The main objectives of this study were 1) to determine the carriage rate, serotype distribution and antibiotic susceptibility patterns of *S. pneumoniae* in oropharyngeal (OP) carrier healthy children of our community, 2) to assess the potential serotype coverage of the recently licensed pneumococcal conjugate vaccines PCV10 and PCV13 against pneumococcal carriage in our population, and 3) to evaluate the reported risk factors for pneumococcal carriage.

2. Materials and methods

2.1. Study population

From April 2004 through March 2006, OP swabs from 502 children aged between 6 months and 6 years of age were obtained. The children were attended for minor surgical procedures at the Outpatient Department of Sant Joan de Déu Hospital. During the study period, this was tertiary-care children's and maternity hospital in Barcelona (Catalonia, Spain) with 345 beds and an average referral population of 210,000 children under 18 years of age (data obtained from the Statistical Institute of Catalonia [Institut d'Estadística de Catalunya], http://www.idescat.net, accessed August 2011]).

The samples were obtained uniformly during the study period except in August, when no cases were recruited because of researcher vacation. Children suffering from fever or acute upper or lower respiratory tract infection were excluded. During the first year of the study period OP swabs were also collected from the patients' mothers.

A structured interview was conducted to elicit the following data: age, gender, number of siblings, passive smoking, relatives with chronic respiratory illness, DCC attendance, and medical history, including underlying disorders, PCV7 status (not vaccinated, partially vaccinated, or vaccination up-to-date for age at the time of enrolment), hospital admissions or ear infections within the previous three months, and recent antibiotic use (defined as use at time of study or in the immediately prior month). No specific information from the patients' mothers was recorded.

Signed informed consent was obtained from the parents or legal guardians of participating children prior to OP swabbing.

2.2. Sampling

One sample from each individual (mother and child) was obtained at the same visit. OP swab specimen was taken by direct inspection of the posterior wall and tonsil regions using rigid cotton-tipped wooden applicators. The swabs were then inoculated as previously described by the WHO working group (O'Brien et al., 2003). In order to minimize possible distortion due to different collection methods, all the specimens were obtained exclusively by the researcher herself, having been trained in advance.

2.3. Bacteriology

Swabs were inoculated onto Columbia Agar with 5% sheep blood and 5.0 μg of gentamicin/ml and were incubated aerobically at 37ºC for 48h. *S. pneumoniae* isolates were identified by standard microbiological procedures. Agar dilution technique was used to determine the minimum inhibitory concentrations (MIC) of penicillin, cefotaxime, erythromycin, tetracycline, levofloxacine, and chloramphenicol. Antibiotic susceptibilities were interpreted according to the 2008 meningeal break points defined in Clinical Laboratory Standards Institute (CLSI) document M100-S18 (CLSI, 2008) so, isolates with MIC to penicillin $\geq$ 0.12μg/ml were defined as penicillin-nonsusceptible *S. pneumoniae* (PNSP). Multidrug resistance was defined as resistance to three or more classes of antimicrobial agents. Serotyping was performed with the Quellung reaction. Pneumococccal isolates were classified as PCV7 serotypes (VT), non-PCV7 serotypes (NVT) (all other serotypes), or non-typable (NT). All strains were sent to the National Pneumococcus Reference Centre (Majadahonda, Madrid, Spain) for determination of both MICs and serotypes.

2.4. Statistical analysis

Sample size calculation was performed according to an estimated carriage prevalence of 25%, an alpha level of 0.05 and a power of 90%. All clinical, epidemiological and microbiological variables analyzed were introduced into a computerized data base (Microsoft Access 97). Quantitative and qualitative variables were tabulated. Statistical studies were carried out using the SPSS program (version 17.0) for Windows. The chi-square test and Fisher's exact test were used for the analysis of qualitative variables. Univariate analysis was performed to determine the relative risk of being a carrier according to different risk factors. The confidence interval was calculated at 95%. Two-tailed tests to determine the significance of risk factors were performed at the 5% significance level. Statistical significance was defined as a P value of $\leq$ 0.05.

3. Results

3.1. Study population characteristics

OP swabs were obtained from 502 children between 6 months and 6 years of age during the study period. Mean age was 36.9 months (standard deviation: 18.8 months) and 317 (63.1%) were males. Three-hundred forty-five (68.7%) children attended DCC and 57 (11.3%) had more than one sibling. One hundred and twelve (22.3%) children had received antibiotic treatment in the previous month. Of 497 patients with available data about PCV7 immunization, 25.3% had received at least one dose; this rate increased from 20.1% (45 of 224 patients) during the first year of the study to 29.7% (81 of 273 patients) during the second year.

3.2. Prevalence of carriage and risk factors

S. pneumoniae was isolated from the oropharynx of 118 of the 502 children, representing an overall colonization rate of 23.5% (95% CI: 19.9- 27.5). This rate ranged from 5.9% (children aged between 6 and 12 months) to 32.3% (children aged between 24 and 36 months). Univariate risk factors for pneumococcal carriage are shown in Table 1. *S. pneumoniae* was isolated from the oropharynx of 6 (2.4%) of the 248 mothers included during the first year of the study; in one case it was also isolated from the child. Because of this low prevalence, no OP swabs were collected from mothers during the second year of the study.

3.3. Serotype distribution and antibiotic susceptibility of the OP isolates in healthy children

A single pneumococcal isolate was recovered from each carrier. Serotyping was performed on 116 (98.3%) of 118 isolates. A total of 31 serotypes were identified, with 6 isolates being NT. The predominant serotypes were 19F (16 cases; 13.8%), 6A (12 cases; 10.3%), 23F (8 cases; 6.9%) and 19A (7 cases; 6.0%) (Fig. 1). Overall, 36 (31.0%) of the isolates were contained in the PCV7, 39 (33.6%) in the PCV10, and 62 (53.4%) in the PCV13.

Carriage of VT pneumococci among PCV7 unvaccinated children was significantly higher than among partially and completely vaccinated children (40.5% vs. 20.0% and 10.5% respectively, $P = 0.009$).

The only mother-child pair of *S. pneumoniae* carriers was colonized with the same serotype (19F). The serotypes isolated in the other 5

Table 1
Demographic and clinical characteristics of the 502 children included in the study. Univariate risk factors for pneumococcal carriage.

Characteristic (n)	N	Carriers (118)No. (%)	Non-carriers (384)No. (%)	*P*	OR (95%CI)
Age ≥ 24 months (502)					
Yes	348	97 (27.9)	251 (72.1)	0.001	2.45 (1.46-4.10)
No	154	21 (13.6)	133 (82.4)		
Gender (502)					
Male	317	68 (21.5)	249 (78.5)	0.155	0.74 (0.48-1.12)
Female	185	50 (27.0)	135 (73.0)		
Daycare attendance (502)					
Yes	345	101 (29.3)	244 (70.7)	<0.001	3.41 (1.96-5.93)
No	157	17 (10.8)	140 (89.2)		
Number of siblings (502)					
≤ 1	445	106 (23.8)	339 (76.2)	0.643	0.85 (0.43-1.67)
> 1	57	12 (21.1)	45 (78.9)		
Underlying disorders[a] (502)					
Yes	119	22 (18.5)	97 (81.5)	0.139	0.68 (0.40-1.14)
No	383	96 (25.1)	287 (74.9)		
PCV7 status (497)					
Not vaccinated	371	85 (22.9)	286 (76.1)	0.442	0.75 (0.43-1.31)
Partially vaccinated	52	10 (19.2)	42 (80.8)		0.60 (0.26-1.41)
Completely vaccinated	74	21 (28.4)	53 (71.6)		reference
Antibiotic last month (502)					
Yes	112	14 (12.5)	98 (87.5)	0.002	0.39 (0.26-0.72)
No	390	104 (26.7)	286 (73.3)		
AOM last 3 months (502)					
Yes	81	18 (22.2)	63 (77.8)	0.766	0.96 (0.52-1.62)
No	421	100 (23.8)	321 (76.2)		
Hospitalisation last 3 months[b] (502)					
Yes	26	0 (0)	26 (100)	0.04	0.93 (0.91-0.96)
No	476	118 (24.8)	358 (75.2)		
Passive smoking (502)					
Yes	256	59 (23.0)	197 (77.0)	0.805	0.89 (0.58-1.35)
No	246	59 (24.0)	187 (76.0)		
Colder months (502)					
Yes	280	60 (21.4)	220 (78.6)	0.218	0.77 (0.51-1.17)
No	222	58 (26.1)	164 (73.9)		
Relatives with CRI (502)					
Yes	30	7 (23.3)	23 (76.7)	0.98	0.99 (0.41-2.37)
No	472	111(23.5)	361 (76.5)		

CI = confidence interval; OR = odds ratio; AOM = acute otitis media; Colder months = October to March; CRI = chronic respiratory illness.
[a] Underlying disorders: metabolic-endocrine (25 cases), neurological disease (22 cases), respiratory disease (19 cases), immunosuppressant (10 cases), alimentary allergy (10 cases), haematological disease non immunosuppressant (9 cases), dermatological disease (7 cases), congenital cardiac disease (5 cases), renal disease (5 cases) and others (7 cases).
[b] Hospitalisation last 3 months: infectious disease (12 cases), decompensated underlying disease (5 cases), minor surgical procedures (4cases), bronchospasm (3 cases), and seizure (2 cases).

mothers colonized were 6A (two cases), and one each of 19A, 23F and 7F.

Forty-four strains (37.9%) were penicillin-nonsusceptible. VT strains were more likely to be PNSP than NVT strains (66.7% vs. 21.6%; $P < 0.001$). PCV7 serotypes accounted for 54.5% of all PNSP isolates. Of note, the most prevalent NVT, 6A and 19A, were mainly penicillin-susceptible (12/12 and 6/7 respectively). The potential PCV10 and PCV13 coverage of PNSP isolates was 54.5% and 56.8%, respectively. Antimicrobial susceptibility is shown in Table 2. No cases of resistance to levofloxacine were identified. A pattern of multidrug resistance was observed in 22 isolates (18.9%).

4. Discussion

In this study, a pneumococcal OP carriage rate of 23.5% was found among healthy children under six years old from our geographical area. This is somewhat lower than the prevalence reported in three previously published Spanish carriage studies; pneumococcal carriage rates of 28% and 36% were found in two studies carried out before the introduction of PCV7 (García de Lomas et al., 1997; Lopez et al., 1999), and 31% in a study published after that (Sánchez-Tatay et al., 2008). Carriage rates observed in children from other industrialised countries ranged from 15% to nearly 70% [Frazao et al., 2005; Petrosillo et al., 2002; Roche et al., 2007; Grivea et al., 2008; Varon et al., 2000). Variations in study population, sampling methods and culture techniques preclude comparison among different studies. For instance, several factors might account for the lower prevalence rates compared with other studies. First, we used OP swabs as a less invasive method than NP swab; however NP sampling has been shown to be somewhat superior to OP in detecting *S. pneumoniae* in paediatric populations, especially in young children (Greenberg et al., 2004). Second, we excluded children with respiratory tract infections, who are more likely to be colonised by *S. pneumoniae* (Dunais et al., 2003). A third factor is that carriage is often evaluated in children attending DCC, which is another reported risk factor for pneumoccocal carriage, whereas the present study included children at a hospital outpatient department.

Among the several risk factors analyzed, there were few differences between the characteristics of carriers and non-carriers, with DCC attendance being the greatest risk factor for pneumococcal carriage detected in our study. This association is well documented; the presence of a large number of susceptible children, crowding, and high rates of viral respiratory tract infections are conditions that favour the development and transmission of pneumococci (Dunais et al., 2003; Sa-Leao et al., 2008).

The extremely low carriage rate concurrently found in the patients' mothers is similar to that described by other authors (Regev-Yochay et al., 2004). It is known that the rate of *S. pneumoniae* carriage is low in adults compared with children; however, because adults are known to carry *S. pneumoniae* for shorter time periods (Melegaro et al., 2004), and because only a single swab specimen was obtained from each individual, the correlation between carriage rates in children and their mothers and the concordance of serotypes may have been partially underestimated.

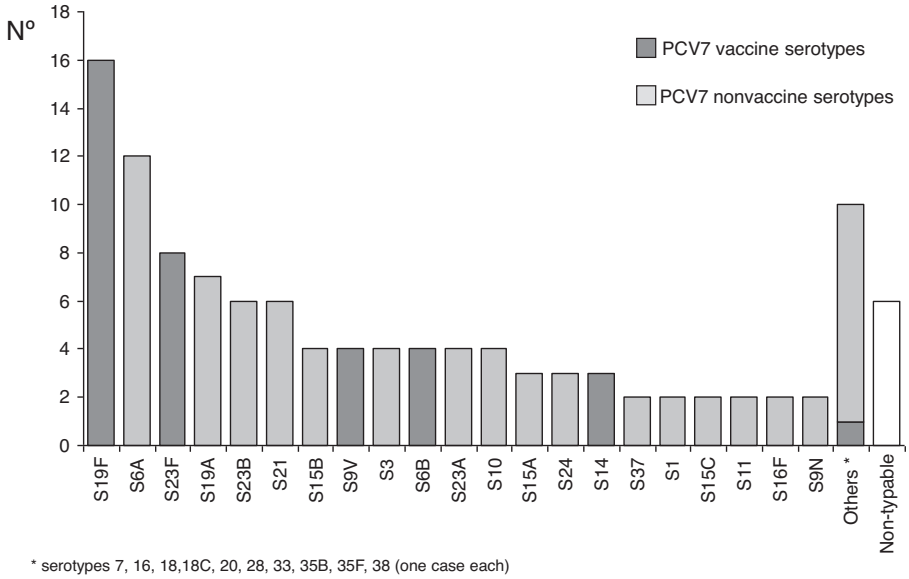

Fig. 1. Distribution of *S. pneumoniae* serotypes from 116 oropharyngeal carriage isolates.

Overall, PCV7 serotypes accounted for 31% of isolates. Similar results were described not only in a Spanish study performed after PCV7 (Sánchez-Tatay et al., 2008) but also in several studies carried out in other countries with low PCV7 coverage (Finkelstein et al., 2003; Grivea et al., 2008) as in our population. In like manner, the serotype distribution found, with 19F, 6A, 23F and 19A serotypes being the most prevalent, was very similar to what was described by these authors.

According to other authors (Shouval et al., 2008), PCV10 extended the serotype coverage by only 2.6%. This limited benefit is due to the low presence of the serotypes added among carriage isolates, in contrast with invasive isolates, with serotypes 1 and 5 being highly prevalent in our population (Muñoz-Almagro et al., 2008).

We detected no differences between PCV7 vaccinated and unvaccinated children in the overall pneumoccocal carriage rate. In contrast, colonization with VT was significantly associated with the absence of PCV7 immunization. These observations are also in accordance with published reports (Gonzalez et al., 2006; Grivea et al., 2008; Sánchez-Tatay et al., 2008).

Table 2
Antimicrobial susceptibility of 116 *Streptococcus pneumoniae* isolates recovered from oropharyngeal swabs from asymptomatic children.

Antimicrobial agent and MIC (μg/ml)	Nº of strains (%)
Penicillin G	
≤0.06	72 (62.1)
≥0.12	44 (37.9)
Cefotaxime	
≤0.5	113 (97.4)
1	3 (2.6)
≥2	-
Erythromycin	
≤0.25	74 (63.8)
0.5	-
≥1	42 (36.2)
Tetracycline	
≤2	72 (62.1)
4	-
≥8	44 (37.9)
Chloramphenicol	
≤4	100 (86.2)
≥8	16 (13.8)

MIC: minimum inhibitory concentration.
NOTE: None of the strains were resistant to levofloxacine, defined as an MIC ≥4 μg/ mL.

The rate of penicillin resistance and its correlation with VT strains were similar to what was described in the other Spanish study performed after the introduction of PCV7 (Sánchez-Tatay et al., 2008), whereas the two Spanish studies performed in the prevacunal age showed clearly higher rates of penicillin resistance (64% and 68%, respectively) (García de Lomas et al., 1997; Lopez et al., 1999). Those results are in agreement with the significant decrease in penicillin resistance observed in Spain in the last decade among the paediatric population (Oteo et al., 2004), with the key factors being the introduction of PCV7 and the decrease in antibiotic use.

Among NVT, penicillin resistance was associated with less prevalent serotypes, whereas the most prevalent serotypes (6A and 19A) during the study period showed greater penicillin sensitivity; for this reason the potential coverage of PNSP isolates by the new pneumococcal conjugate vaccines was very similar to PCV7. Nevertheless, in recent years some authors have sounded the alarm about the emergence of IPD caused by resistant NVT, in special serotype 19A (Muñoz-Almagro et al., 2008; Techasaensiri et al., 2010).

This study has some limitations. First, we included patients attending the hospital Outpatient Department, so the results obtained could not be representative of our entire pediatric population. Second, as previously indicated, not only the choice of the OP for swabbing, but also the performing of a single swab per patient may have led to underestimate the pneumococcal carriage prevalence. Although if several samples were simultaneously obtained the detection of *S. pneumoniae* could have been somewhat optimized, a single swab was obtained to diminish the discomfort to participating children, according to similar studies published. In adition, in the present study serotyping was performed with the Quellung reaction. However, it has been reported that the most effective technique for detecting pneumococcal carriers is multiplex- polymerase chain reaction (PCR) (Ercibengoa et al., 2012). Unfortunely, we did not have this technique available in the Laboratory Department during the study period. Third, even though significant differences were detected in the serotype distribution among carriers according to their PCV7 status, the low PCV7 coverage was insufficient for analysis of the impact of this vaccine on serotype distribution and antibiotic susceptibility.

In conclusion, a pneumococcal carriage rate of 23.5% was found among healthy children in our geographical area. NVT were predominant whereas antibiotic resistance was significantly associated with VT. Nevertheless, because of the low PCV7 vaccine coverage among our population, continuing careful surveillance is essential to

evaluate the long-term impact of pneumococcal conjugate vaccines on carriage, serotype distribution and antibiotic susceptibility.

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Pneumococcal Serotypes Causing Acute Otitis Media Among Children in Barcelona (1992–2011): Emergence of the Multiresistant Clone ST320 of Serotype 19A

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Background: There is scarce information about changes in serotypes and clonal types of *Streptococcus pneumoniae* causing acute otitis media (AOM) in recent years, particularly in European countries.

Methods: Pneumococcal serotypes and clones from *S. pneumoniae* strains isolated from children with AOM who were attended at Hospital Sant Joan de Déu, Barcelona (1992 to 2011), were studied. Heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in June 2001. We defined 3 periods: prevaccine period 1992 to 2001, early vaccine period 2002 to 2006 and late vaccine period 2007 to 2011.

Results: There were 376 pneumococcal strains causing AOM, and 373 (99.2%) of them were serotyped. AOM caused by PCV7 serotypes declined significantly: 161 of 245 (65.7%) episodes in 1992 to 2001 versus 22 of 67 (32.8%) in 2002 to 2006 versus 8 of 61 (13.1%) in 2007 to 2011 $P < 0.001$. In the last period (2007 to 2011), the potential serotype coverage for the PCV10 was 16.4% and for the PCV13 was 68.9% ($P < 0.001$). Serotype 19A increased from 5.7% in 1992 to 2001 to 42.6% in 2007 to 2011 ($P < 0.001$). Among strains with penicillin minimal inhibitory concentration ≥0.12 μg/mL (n = 241), serotype 19A rose from 2.3% in the first period to 57.9 % in the last period ($P < 0.001$). The clonal-type ST320 was initially detected in 2005, and in the period 2007 to 2011, the ST320 was found in 72.7% of nonsusceptible serotype 19A isolates.

Conclusions: Among children with AOM, a rapid expansion of the multiresistant clone ST320 expressing serotype 19A has been observed in Barcelona. The implementation of PCV13, which includes this serotype, may decrease the prevalence of AOM and reduce antimicrobial resistance.

Key Words: otitis, *Streptococcus pneumoniae*, multi locus sequence typing, serotypes, children

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Acute otitis media (AOM) is a common infection in children, and *Streptococcus pneumoniae* remains its main etiologic agent.[1] Even in the current pneumococcal conjugate vaccine (PCV) era, AOM remains highly prevalent, accounting for a huge number of episodes worldwide. At present, annually >1,500,000 cases of AOM occur in the United States at an estimated cost of 440 million US dollars.[2]

The heptavalent (PCV7) has proven to be safe, immunogenic and effective in preventing AOM in children and other pneumococcal infections caused by vaccine serotypes in children and adults (reflecting herd protection).[3–6] Nonetheless, emergence of nonvaccine serotypes[7–10] has reduced the potential benefits of PCV7 routine vaccination. Additional concerns have arisen about the increase of antimicrobial resistance in nonvaccine serotypes.[11–14] In this respect, serotype 19A has particularly become one of the principal emergent serotypes with diminished susceptibility to antimicrobial agents.[15–20]

Even though geographical and temporary differences in the main resistant clonal types of *S. pneumoniae* and their associated serotypes have been widely documented, data on this subject primarily refer to invasive pneumococcal disease and nasopharyngeal carriers. However, little is known about the clonal composition and especially about the evolution of antimicrobial susceptibility related to serotypes and clonal types involved in AOM. This scarce information, together with the empirical approach adopted to treat the disease, make it necessary to know the changes in antibiotic resistance and serotypes causing pneumococcal AOM.

Considering the change in the distribution of serotypes in invasive pneumococcal disease after implementation of the heptavalent vaccine, presumably this change has taken place also in episodes of AOM. For this reason, the main goal of this study was to investigate changes in serotype distribution and antimicrobial resistance of *S. pneumoniae* causing AOM in children during a 20-year period. A secondary goal was to provide baseline information about the clonal composition and serotype distribution of resistant isolates before the introduction of the new 10-valent and 13-valent conjugate vaccines.

MATERIALS AND METHODS

Setting and Definitions

We conducted a prospective study including all *S. pneumoniae* strains isolated from children with (AOM) attended at Hospital Sant Joan de Déu, located in the Barcelona area (Catalonia, Spain), from 1992 to 2011. Since 1989, all strains of *S. pneumoniae* isolated from clinical samples at the Clinical Microbiology Department have been prospectively frozen at –80°C and several variables have been routinely recorded, including type of sample, age, sex, diagnosis, serotype and antimicrobial susceptibility. All data were collected following the guidelines of the hospital's ethics committee.

A case of pneumococcal AOM was diagnosed when the patient showed signs/symptoms of the disease together with isolation of *S. pneumoniae* from spontaneous ear secretions or samples obtained by tympanocentesis. Only 1 episode per patient was considered.

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PCV7 was introduced in Spain in 2001. Two newer conjugate vaccines that widen PCV7 serotype spectrum have recently been available: a 10-valent conjugate vaccine (PCV10), which includes PCV7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, plus serotypes 1, 5 and 7F, and PCV13 (PCV10 plus additional serotypes 3, 6A and 19A). Although utilization of conjugate vaccines is recommended by the Spanish Association of Pediatrics,[21] the regional public health system has not included them in routine vaccination programs yet. A moderate PCV7 uptake of 50% was estimated among the pediatric population of Catalonia in 2007.[22]

Microbiological Identification, Serotyping and Antimicrobial Susceptibility

Isolates were identified in our laboratory by standard microbiological methods that included Gram strain morphology, optochin sensitivity test and bile solubility test, antigenic test and biochemical methods. The antimicrobial susceptibility was tested by a microdilution method. Isolates were serotyped by a published multiplex-polymerase chain reaction 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/B/C, 23A and 23F).[23] A specific polymerase chain reaction to detect serotype 6C was used to distinguish serotype 6A and 6C.[24]

All strains were also sent to the National Pneumococcus Reference Center of Majadahonda (Madrid, Spain), to complete serotype study by Quellung reaction and determine the minimal inhibitory concentrations (MICs) of penicillin and other antibiotics by Agar dilution technique. Antibiotic susceptibilities were defined according to the 2008 breakpoints suggested by the Clinical Laboratory Standards Institute.[25] Isolates with intermediate or high-level resistance were defined as nonsusceptible. We adopted oral penicillin breakpoints (susceptible ≤2 μg/mL, intermediate 4 μg/mL and resistant ≥8 μg/mL) for 2 reasons: first, for the purpose of epidemiological surveillance; second, on the basis that treatment of otitis is mainly oral. Multidrug resistance was defined as nonsusceptibility to 3 or more antimicrobial agents

Clonal Analysis

Nonsusceptible penicillin strains isolated during the last 5 years of study (when vaccine coverage in our population was estimated to be >50%) were included for clonal analysis by multi locus sequence typing. In addition, given that serotype 19A was the main emergent serotype detected in this study, all nonsusceptible penicillin serotype 19A strains were studied by multi locus sequence typing. The assignment of alleles and sequence types (STs) 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. STs that shared 6 of 7 allelic (single locus [SLV]) variants were considered a clonal complex.

Statistical Analysis

The study was divided into 3 periods: prevaccine period 1992 to 2001, early vaccine period (2002 to 2006), when vaccine coverage in our population was estimated to be <50%,[8] and late vaccine period (2007 to 2011), with an estimated vaccine coverage >50%.[22]

We used the χ^2 test or Fisher exact test to compare proportions. Nonparametric Kruskal–Wallis test was utilized to compare differences in susceptibility of strains to different antimicrobial agents for each age group. Statistical analyses were performed using Statistical Package for Social Sciences software for Windows, version 18. *P* values <0.05 were considered to be statistically significant.

RESULTS

During the study period, 376 AOM episodes caused by *S. pneumoniae* occurred. There were 220 (58.5%) males and 156 (41.5%) females, with a median age of 12 months (range: 1 month–11 years).

Serotype Distribution

Three hundred seventy-three of 376 (99.2%) strains were serotyped, and 41 different serotypes were found. AOM caused by PCV7 serotypes declined significantly: 161 of 245 (65.7%) episodes in 1992 to 2001 versus 22 of 67 (32.8%) in 2002 to 2006 versus 8 of 61 (13.1%) in 2007 to 2011 $P < 0.001$. In the last period (2007 to 2011), the potential serotype coverage for the PCV10 was 16.4% and for the PCV13 was 68.9% ($P < 0.001$).

Table 1 shows the most prevalent pneumococcal serotypes causing AOM over the study period. During the first period, the 3 main serotypes detected were serotype 19F (22.4%), serotype 14 (13.9%) and serotype 6B (13.5%). During the second period, serotype 19A was the most frequently found serotype (31.3%), followed by serotype 19F (14.9%), 23F (7.5%) and 6A (7.5%). In the last period, serotype 19A (42.6% of total isolates) markedly showed to be the most predominant, followed by serotype 14 (6.6%) and serotypes 3, 6A and 6C and 23B each one with a rate of 4.9%. In fact, a significant increase of proportion of serotype 19A was detected: from 5.7% of total serotypes in the first period up to 42.6% in the last period ($P < 0.001$).

Antimicrobial Susceptibility and Clonal Study

Overall, the percentage of nonsusceptible strains according to oral penicillin breakpoints (≥0.12 μg/mL) was higher along the study period, 241 of 376 isolates (64.1%). The rate of nonsusceptible isolates varied from 69.9% (172 of 246 strains) in the first period to 45.6% (31 of 68 strains) in the second period and 61.3% (38 of 62 strains) in the last period ($P = 0.001$).

One hundred sixty-four of 241 (68.0%) strains with MIC ≥0.12 μg/mL were PCV7 serotypes. The proportion of PCV7 serotypes among strains with MIC ≥0.12 μg/mL decreased from 82.6% (142 of 172 strains) in the first period to 48.4% (15 of 31 strains) in the second period and 18.4% (7 of 38 strains) in the last period ($P < 0.001$). The proportion of PCV10 serotypes was exactly the same than that described for PCV7 serotypes in the last period of study (18.4%) while proportion of PCV13 serotypes was significant higher than 78.9%. Of note, serotype 19A rose from 2.3% during the first period to 29 % during the second period and 57.9% in the last period of study ($P < 0.001$). Figure 1 shows the serotype distribution of isolates with MIC ≥ 0.12 μg/mL to penicillin in the 3 periods of study.

A MIC ≥ 2 μg/mL was detected in 72 isolates, and 49 (68%) were PCV7 serotypes; the rest were 18 isolates serotype 19A, 3 isolates 6A, 1 isolate 11A and 1 isolate 9N. With regard to serotype 19A, it increased significantly from 0% (0 of 48 isolates) in the first period to 54.5% in the second period (6 of 11 isolates) and to 92.3% (12 of 13 isolates) during the last period ($P < 0.001$). Worrisome, among the 8 strains with MIC = 4 μg/mL, 5 of them were serotype 19A whereas the rest were 3 strains serotype 23F. No strains with MIC ≥ 8 were found in this study.

Table 2 shows antimicrobial resistance according to Clinical Laboratory Standards Institute breakpoints for penicillin and other antimicrobials. Of note, 43 of 62 strains (69.4%) showed multidrug resistance in the last period of study. Thirty-four of these 43 strains (79.1%) expressed PCV13 serotypes.

All strains (n = 38) with MIC ≥ 0.12 μg/mL to penicillin isolated during the last period of the study were included for clonal analysis by multi locus sequence typing. A total of 19 different

TABLE 1. The Most Prevalent Pneumococcal Serotypes Causing AOM During the 3 Periods of Study

	1992–2001		2002–2006		2007–2011	
Serotype	No. of Isolates	Percentage	No. of Isolates	Percentage	No. of Isolates	Percentage
PCV7	161	65.7	22	32.9	8	13.1
19F	55	22.4	10	14.9	2	3.3
14	34	13.9	3	4.5	4	6.6
6B	33	13.5	3	4.5	0	0
23F	26	10.6	5	7.5	1	1.6
9V	8	3.3	0	0	0	0
4	3	1.2	0	0	0	0
18C	2	0.8	1	1.5	1	1.6
Non-PCV7	84	34	45	67.3	53	86.3
5	2	0.8	1	1.5	0	0
1	1	0.4	0	0	1	1.6
7F	0	0	2	3	1	1.6
6A	29	11.8	5	7.5	3	4.9
19A	14	5.7	21	31.3	26	42.6
3	15	6.1	3	4.5	3	4.9
22F	3	1.2	0	0	0	0
9N	3	1.2	0	0	0	0
NT	3	1.2	0	0	1	1.6
20	2	0.8	0	0	0	0
10F	1	0.4	0	0	0	0
12F	1	0.4	0	0	1	1.6
13	1	0.4	0	0	0	0
15F	1	0.4	0	0	0	0
18F	1	0.4	0	0	0	0
21	1	0.4	0	0	1	1.6
23A	1	0.4	0	0	1	1.6
23B	1	0.4	0	0	3	4.9
31	1	0.4	0	0	0	0
9A	1	0.4	0	0	0	0
15A	0	0	4	6	1	1.6
10A	0	0	2	3	1	1.6
24	0	0	2	3	1	1.6
16	0	0	2	3	0	0
10	0	0	1	1.5	0	0
13B	0	0	1	1.5	0	0
17	0	0	0	0	1	1.6
27	0	0	1	1.5	0	0
35B	0	0	0	0	1	1.6
6C	2	0.8	0	0	3	4.9
15B	0	0	0	0	2	3.3
11A	0	0	0	0	1	1.6
11F	0	0	0	0	1	1.6

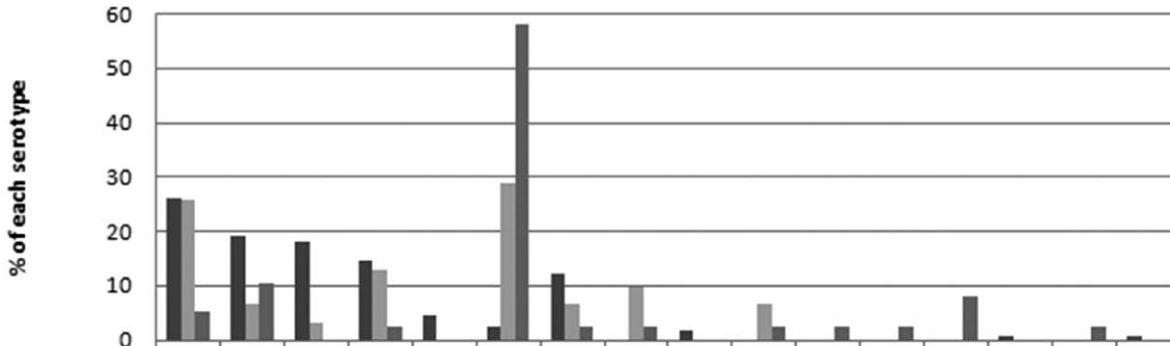
STs were detected. By far, the most frequent ST was ST320, found in 16 strains (38.1 %) expressing all of them serotype 19A. The second most prevalent ST was ST156 detected in 4 strains (9.5%) expressing serotype 14. CC230 (which included ST276 and ST230) was also detected in 4 strains, 3 expressing serotype 19A and 1 expressing serotype 24. CC2013 (including ST5195), all expressing serotype 19A, and ST2372, all expressing serotype 23B, were detected in 3 strains each. CC63, which included ST2100, was detected in 1 strain 15A and another 19F. The rest of ST (1 strain each) was detected in the following serotypes: ST81 (serotype 23F), ST62 (serotype 11F), ST113 (serotype 18C), ST344 (nontypeable), ST432 (serotype 21), ST558 (serotype 35B), ST1545 (serotype 19F), ST4833 (serotype 12F), ST5740 (serotype 6A) and ST6521 (serotype 11A). It is to be highlighted that the 3 last STs and ST5195 and ST2372 were observed in our geographical area for the first time.

Clonal Study of Serotype 19A Pneumococci Nonsusceptible to Penicillin

Due to the predominance of serotype 19A among nonsusceptible penicillin strains (22 of 38 isolates in the last period of study), clonal analysis of all nonsusceptible serotype 19A isolates was performed along the study (n = 35). Overall 5 different clonal types appeared: ST320 (n = 18), ST276 (n = 9), ST2013 (n = 4), ST81 (n = 3) and ST5195 (n = 1). eBurst analysis considered ST2013 and ST5195 as clonal complexes sharing 6 of 7 alleles. Interestingly, ST320 was detected for the first time in 2005 and a rapid expansion of this clonal type has been observed after its introduction, increasing from 22.2% (2 of 9 strains) during 2002 to 2006 to 72.7% (16 of 22 strains) during the late vaccine period (2007 to 2011) ($P < 0.001$). Moreover, as shown in Figure 2, a clonal replacement was observed in these isolates comparing the prevaccine period (with ST81 as the most prevalent ST) with the vaccine period (with ST320 as the most prevalent ST).

DISCUSSION

The results obtained in this study support previous research that has showed a progressive declined in the number of AOM caused by PCV7 serotypes after the introduction of the vaccine and an increase of the strains resistant to antibiotics due to the emergence of non-PCV7 serotypes, taking into account that in



	19F	14	6B	23F	9V	19A	6A	15A	9N	24	NT	35B	23B	15F	11A	6C
1992-2001	26,2	19,2	18	14,5	4,7	2,3	12,2	0	1,7	0	0	0	0	0,6	0	0,6
2002-2006	25,8	6,5	3,2	12,9	0	29	6,5	9,7	0	6,5	0	0	0	0	0	0
2007-2011	5,3	10,5	0	2,6	0	57,9	2,6	2,6	0	2,6	2,6	2,6	7,9	0	2,6	0

FIGURE 1. Percentage of different serotypes among strains with MIC ≥ 0.12 μg/mL in the 3 periods of study.

TABLE 2. Antimicrobial Susceptibility of Pneumococcal Strains Causing AOM

	1992–2001	2002–2006	2007–2011	
MIC (mg/L)	No. of Strains (%) n = 246	No. of Strains (%) n = 86	No. of Strains (%) n = 44	*P*
Penicillin G (meningeal breakpoints) and oral penicillin V breakpoints				
≤0.06	74 (30.1)	37 (54.4)	24 (38.7)	0.001
≥0.12	172 (69.9)	31 (45.6)	38 (61.3)	
Penicillin G (nonmeningeal breakpoints)				
≤2	244 (99.2)	67 (98.5)	57 (91.9)	0.002
4	2 (0.8)	1 (1.5)	5 (8.1)	
8	0 (0)	0 (0)	0 (0)	
Cefotaxime (meningeal breakpoints)				
≤0.5	173 (70.3)	58 (85.3)	37 (59.7)	<0.001
1	69 (28)	10 (14.7)	11 (17.7)	
≥2	4 (1.6)	0 (0)	14 (22.6)	
Cefotaxime (nonmeningeal breakpoints)				
≤1	242 (98.4)	68 (100)	48 (77.4)	<0.001
2	4 (1.6)	0 (0)	14 (22.6)	
≥4	0 (0)	0 (0)	0 (0)	
Erythromycin				
≤0.25	121 (49.2)	27 (39.7)	29 (46.8)	0.637
0.5	1 (0.4)	0 (0)	0 (0)	
≥1	124 (50.4)	41 (60.3)	33 (53.2)	
Tetracycline				
≤2	82 (33.3)	27 (39.7)	29 (46.8)	0.265
4	4 (1.6)	0 (0)	1 (1.6)	
≥8	160 (65)	41 (60.3)	32 (51.6)	
Chloramphenicol				
≤4	136 (55.3)	60 (88.2)	59 (95.2)	<0.001
≥8	110 (44.7)	8 (11.8)	3 (4.8)	
Multidrug resistance				
0	107 (43.5)	39 (57.4)	19 (30.6)	0.009
1	139 (56.5)	29 (42.6)	43 (69.4)	

the prevaccine period non-PCV7 serotypes were more susceptible than PCV7 serotypes and a decrease in antibiotic resistance would have been expected.[14] Specially, a significant increase of strains nonsusceptible to Cefotaxime (which translate to Ceftriaxone) and multidrug-resistant strains was detected in the last period of the study. Such increase was mainly attributable to the emergence of serotype 19A as otopathogen in our geographical area as had already been described in diverse countries such as Australia, Italy, Portugal, United Kingdom and United States.[8,26–30] Of note, this emergence had also been documented in some countries before the introduction of conjugate vaccines that highlights the likelihood of a multifactorial cause for serotype 19A emergence.[31,32] In this respect, a genome-wide dissection of selected globally emergent multidrug-resistant serotype 19A isolates from Canadian patients has recently reported new information. The results of Canadian study suggest that PCV7 vaccine selective pressure, antibiotic selection pressure and propensity for genetic change of *S. pneumoniae* could be related to the emergence of multidrug-resistant serotype 19A.[33]

Additionally, in this study, we observed a replacement in the clonal composition of multiresistant serotype 19A strains with a statistically significant increase of ST320 clone. The replacement was so extensive that during the last year of the study this clone ST320 was detected in almost all 19A isolates with diminished susceptibility to penicillin. On the other hand, multiresistant clone ST81, which had been highly prevalent in Spain during the prevaccine era,[8] did not appear during the vaccine period. The main clonal type detected in the present study showed diminished susceptibility to oral β-lactamic antibiotics and completely resistant to macrolides. Therefore, high doses of oral amoxicillin or i.m. ceftriaxone should be recommended for treating patients with suspected pneumococcal AOM. Of note, it has been reported that ST320 expressing serotype 19A may continue producing symptoms even with a high dose of β-lactamic antibiotic[34], and moreover, a new clone expressing serotype 19A (clonal-type ST2722) has been detected as resistant to all FDA-approved antibiotics for the treatment of AOM in children, which has important clinical consequences.[35] Therefore, prevention of disease caused by 19A serotype is necessary to avoid an important health problem. In our country, it is better to use PCV13 instead of PCV10, due to the lack of 10-valent vaccine against serotype 19A.

In conclusion, a serious concern arises about the rapid expansion of multiresistant serotype 19A, regardless of the causative factors of this change. Due to the rapid and global expansion of pneumococcal multiresistant clones expressing serotype 19A, PCV13 vaccine should promptly be introduced in routine vaccination programs. A rapid implementation of the new vaccine and appropriate use of antibiotics could contribute to reduce the burden of disease and antimicrobial resistance in Spain and other countries. However, according to the lessons learned from PCV7 vaccine spread, the use of PCV13 could be accompanied by an

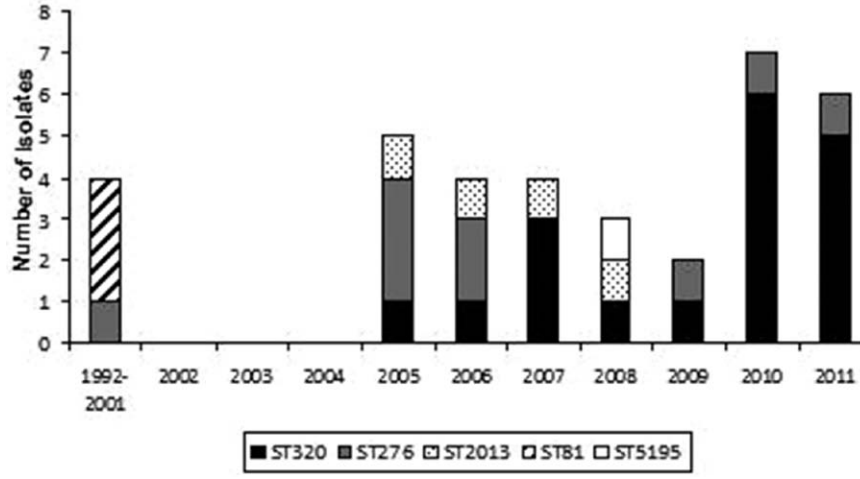


FIGURE 2. Pneumococcal AOM in children. Clonal distribution of serotype 19A isolates with MIC > 0.12 μg/mL along the study period.

unpredictable increases in non-PCV13 serotypes in healthy carriers and likely also as cause of disease. Consequently, it is crucial to maintain surveillance of pneumococcal serotypes and clones after the introduction of 13-valent vaccine in order to adjust prophylaxis and treatment strategies with up-to-date knowledge.

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Erratum

Metacarpal osteomyelitis and chronic granulomatous disease: ERRATUM

In the letter appearing on page 196 of volume 32, issue 2, the order of authors was incorrect. The author listing should appear as follows Wassim Zribi, MD,* Makram Koubaa, MD,† Zoubeir Ellouze, MD,* Dorra Lahiani, MD,† Mounir Ben Jemâa, MD,† Hassib Keskes, MD*. The affiliations are as follows: *Department of Orthopaedic Surgery, Habib Bourguiba University hospital, Sfax, Tunisia; and †Department of Infectious Diseases, Hedi Chaker University Hospital, Sfax, Tunisia.

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High prevalence of genetically-determined mannose binding lectin deficiency in young children with invasive pneumococcal disease

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Abstract

Susceptibility to invasive pneumococcal disease (IPD) correlates with age, younger children being the group with the highest burden of disease. The relevance of the innate immune response and particularly the role of mannose-binding lectin (MBL) in combating IPD is not well known. This is a 2-year prospective study (February 2011 to March 2013) including patients with IPD who attended two hospitals from Catalonia, Spain. Variables including attack rate of pneumococcal serotype (high or low invasive potential serotypes) and genotypes associated with low serum MBL levels were recorded. One hundred and forty-seven patients were included in the study. One hundred and two (69.4%) patients were children or adolescents <18 years and 45 (30.6%) were adults. Overall, low-MBL genotypes (O/O; XA/O) were detected in 23 (15.6%) patients. Children <2 years showed a higher frequency of low-MBL genotypes compared with other patients (31.0% vs. 11.9%; $p = 0.031$). Further sub-analysis revealed a higher proportion of low-MBL genotypes in children <2 years with IPD caused by opportunistic or low-attack-rate serotypes when compared with older patients (46.2% vs. 13.2%; $p = 0.02$). However, no statistically significant differences between the two groups were observed when including patients infected with invasive or high-attack-rate serotypes (18.8% vs. 10.0%; $p = 0.59$). Our data suggest that young children with a genetically determined low-MBL production are at a higher risk of developing IPD, particularly that caused by opportunistic or low-attack-rate pneumococcal serotypes.

Keywords: molecular methods, paediatrics, pneumococcal disease, *Streptococcus pneumoniae*
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Introduction

Invasive pneumococcal disease (IPD) is a serious health problem in children and adults, and causes almost one million childhood deaths worldwide every year [1]. *Streptococcus pneumoniae* usually colonizes the nasopharynx of healthy children but is less frequently found as a colonizer in adults. It is estimated that most children are colonized by pneumococcus

at least once during the first 2 years of life and nasopharyngeal colonization is the first step towards development of mucosal and invasive diseases [2]. Further spread of pneumococcus to the bloodstream and other normally sterile sites occurs less often. However, young children, young adults with immunosuppressive and chronic conditions and older adults are at higher risk of IPD. The complex interaction between impaired host factors and the presence of virulence determinants of the pneumococcus may be responsible for developing IPD [2].

The main virulence factor for pneumococcus is the polysaccharide capsule, with more than 94 serotypes that cause varying rates of carriage and IPD. Some of these serotypes have 'low attack rate' and are frequently detected in carriers. These so-called 'opportunistic serotypes' are more prevalent in children <2 years old, elderly people and patients with co-morbidities [3]. In contrast, serotypes with a 'high attack rate', also called 'high-invasive potential serotypes', are seldom detected in carriers and often cause IPD, particularly in older children and adults without co-morbidities [4,5]. Intriguingly, serotypes with a high attack rate, such as serotypes 1, 5 or 7F, have been associated with a less complicated course of disease and lower mortality rates than opportunistic serotypes [6,7],whereas serotypes with a low attack rate have been related to high mortality and more serious clinical manifestations, such as meningitis and sepsis [8].

Mannose-binding lectin (MBL) is a serum protein of the innate immune system, which recognizes pathogen structures, mainly of a carbohydrate nature. It can then promote opsonophagocytosis of a wide range of microorganisms and subsequent antibody-independent complement activation [9,10]. It is considered a pre-antibody that has a relevant defensive role in the first period of life, when an immature adaptive immune system still exists. The serum levels of MBL are genetically determined as a consequence of single nucleotide polymorphisms (SNPs) embedded into the promoter and the exon 1 of the human *MBL2* gene [11]. Homo- and heterozygous combinations of those SNPs give rise to different genotypes responsible for high (A/A, XA/A), intermediate (O/A, XA/XA) or low (O/O, XA/O) serum MBL levels [12]. Previous reports indicate that genetically-determined MBL deficiency is relatively frequent in all human populations analyzed (ranging from <15% in Caucasian populations to 20% in sub-Saharan African populations) [13,14]. This deficiency has been linked to increased susceptibility to infectious diseases, including those caused by pneumococcus [15]. Nonetheless, this hypothesis remains controversial because some studies have not observed a significant association of MBL deficiency with the development of IPD [16].

The aim of this study was to evaluate the prevalence of genotypes responsible for low serum MBL levels in patients with IPD according to age group and serotype attack rate characteristics. This information could be useful for designing strategies for prevention and personalized treatment of patients based on previous analysis of host–pathogen interactions.

Patients and Methods

Participant recruitment

This is a prospective study that includes all patients with IPD who attended two medical centres (Hospital Sant Joan de Déu and Hospital de Mataró) from 1 February 2011 to 1 March 2013. The Hospital Sant Joan de Déu (HSJD) is a 360-bed referral paediatric centre located in the metropolitan area of Barcelona, which annually captures around 17% of all hospitalizations (*c.* 200 000 children) from the population <18 years in Catalonia (Spain). The Hospital de Mataró (HM) is a public general hospital that covers a catchment area of 400 000 inhabitants from the Catalonian area of Maresme.

Only one episode (the first) per patient was included in the study sample. Patients with functional deficit of classical or alternative pathways of complement activation were excluded from the study, as well as patients with immunocompromised conditions (HIV infection, immunoglobulin deficit), cystic fibrosis, bronchiectasis or cerebrospinal leak.

Demographic and clinical variables including age, sex, ethnicity, IPD risk factors, pneumococcal vaccination status, pneumococcal serotypes and their invasiveness potential, MBL production levels, clinical diagnosis, course of disease, length of hospital stay (LOS) and admission to intensive care unit (ICU) were registered for each episode.

The study was performed following the guidelines of the Ethics Committees of Hospital Sant Joan de Déu and Hospital de Mataró.

Microbiological and immunological methods

Invasive pneumococcal disease was defined as the presence of clinical findings of infection (which were used for classification of disease) together with isolation of *Streptococcus pneumoniae* and/or DNA detection of the *pneumolysin* (*ply*) gene and an additional capsular gene of *S. pneumoniae* by real-time PCR in plasma, cerebrospinal fluid or any other sterile fluid. All pneumococcal isolates were identified by standard microbiological methods. DNA detection of the *pneumolysin* (*ply*) gene by real-time PCR in normal sterile fluids was performed according to a previously reported assay [17]. Serotyping of strains isolated by culture was carried out by a molecular

technique based on automated fluorescent fragment analysis, which allows differentiation of 40 serotypes [18]. Detection of pneumococcal serotypes in culture-negative clinical samples was performed by multiplex real-time PCR methodology [19]. Both molecular techniques were performed at the Molecular Microbiology Department of Hospital Sant Joan de Déu, which operates as the regional support laboratory for pneumococcus surveillance in Catalonia. Quellung reaction performed at the National Center for Microbiology (Majadahonda, Madrid) was used to complete serotyping in strains isolated by culture.

Serotypes were classified according to the studies of Brueggemann [4] and Sleeman [5]: 1, 4, 5, 7F, 9V, 14, 18C and 19A were considered to have a 'high attack rate' whereas the remainder were considered to be non-invasive or opportunistic serotypes with a 'low attack rate'.

MBL2 polymorphism analysis

DNA extraction, amplification and genotyping of *MBL2* were carried out as previously described [20]. Six single nucleotide polymorphisms (SNPs) in the 5′-flanking/promoter region (−550 G/C −221 C/G, 4 C/T) and exon 1 (codon 52 CGT/TGT, codon 54 GGC/GAC and codon 57 GGA/GAA) of the *MBL2* gene were analysed using a polymerase chain reaction (PCR) and sequence-based typing (SBT) technique. The SNPs at codons 52, 54 and 57 are named D, B and C variants, respectively, and are major determinants of serum MBL levels [10,11]. These variants are collectively named O, while A indicates the wild-type variant. The SNPs at positions −551 (H/L), −221 (X/L) and +4 (P/Q) also influence serum MBL levels in individuals with the wild-type A variant [16]. However, the functional effects of H/L and P/Q SNPs appear to be minor compared with L/X, X being the allele associated with lower MBL expression. Accordingly, haplotype combinations O/O and O/XA were considered as low-MBL producing genotypes, O/A and XA/XA as intermediate-producing, and A/A and XA/A as high-producing genotypes.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Sciences software (SPSS Statistics for Windows, version 20.0, IBM Corp., Chicago, IL, USA). Continuous variables were summarized as means and standard deviations (SDs) or as medians and interquartile ranges (IQR, 25th to 75th percentile) according to their homogeneity. Categorical variables were compared with the chi-squared test or the Fisher's exact test (two-tailed) when appropriate. Continuous variables were compared with the Mann–Whitney *U*-test or Student *t*-test according to their homogeneity. Significance was set at a two-sided *p*-value of <0.05 for all statistical analyses. *p*-values were corrected according to the Holm method for multiple comparisons. An eligible population of 170 subjects was expected to be collected in the two hospitals during the study period, according to incidence rates of previous years. Based on results described in previous studies, proportions of low vs. medium-high MBL levels were assumed to be 15% and 85%, respectively, while proportions of IPD caused by opportunistic serotypes vs. high-attack serotypes were assumed to be 50% and 50%. The confidence level was set at 95% (two-tailed), precision of confidence interval at ±3%, and rate of subjects not meeting inclusion criteria at 10%. It was calculated that the minimum sample size to be recruited for the study needed to include 164 subjects from the eligible population.

Results

A total of 203 IPD episodes among 200 patients were recorded in the two institutions. Of these, 36 patients did not give consent to participate in the study (18%) and 17 did not meet inclusion criteria (two patients with cerebrospinal leak, three patients with HIV infection, and 12 patients with immunosuppression treatment). Thus, the final study sample comprised 147 patients with IPD.

Eighty-five patients (57.8%) were recruited at Hospital Sant Joan de Déu and 62 (42.2%) at Hospital de Mataró. The predominant gender was male (85 patients; 57.8%) and the predominant ethnic group was Caucasian ($n = 110$; 74.8%). Children or adolescents <18 years of age were also the predominant age group ($n = 102$; 69.4%). The median age of paediatric patients was 2.9 years (IQR, 1.7–5.3 years) while the median age of adults was 54.0 years (IQR, 47.1–77.6 years). Pneumonia was the most frequent clinical diagnosis ($n = 125$; 85.0%); 66 subjects had complicated pneumonia. This was followed by meningitis ($n = 12$; 8.2%) and bacteraemia/sepsis ($n = 10$; 6.8%). One hundred and thirty-seven (93.2%) patients required hospitalization, with a median LOS of 9.0 days (IQR, 6.0–14.0), and 25 (17.0%) patients required admission to the Paediatric Intensive Care Unit (PICU). Only 45 (30.6%) patients had previously been vaccinated; 15 (10.2%) suffered sequelae and three adults died.

Sixty-five (44.2%) episodes were confirmed only by PCR, 61 (41.5%) by culture and 21 (14.3%) by both PCR and culture. Overall, the rank order of serotypes was serotype 1 ($n = 36$; 24.5%), serotype 3 ($n = 22$; 15.0%), serotype 19A ($n = 11$; 7.5%) and serotype 7F ($n = 10$; 6.8%). Serotypes included in the 13-valent pneumococcal conjugate vaccine were found in 74 (72.5%) of 102 children and adolescents younger than 18 years and in 21 (46.7%) of 45 adults. Serotypes with a high attack rate were identified in 66 (44.9%) episodes.

Table 1 shows the MBL2 genotype frequencies found in this study. A genotype associated with low production of MBL (O/O; XA/O) was detected in 23 (15.6%) patients, while genotypes associated with intermediate and high MBL production were detected in 45 (30.6%) and 79 (53.7%) patients, respectively. Table 2 shows demographic, clinical, microbiological and genotypical variables of patients according to age group.

Low-MBL genotypes were not found to be significantly associated with the variables of sex, ethnicity, serotype invasiveness and PICU admission. A higher proportion of low-MBL genotypes was observed among patients with meningitis in comparison with other clinical presentations (33.3% vs. 14.1%) but this tendency did not reach a statistically significant value ($p = 0.191$). Children younger than 2 years showed a significantly higher frequency of low-MBL genotypes compared with the other patients (31.0% vs. 11.9%; $p = 0.031$). Nevertheless, correction for multiple comparisons did not confirm significance of this association (corrected p-value = 0.186). Data regarding associations between variables are presented in Table 3.

A sub-analysis of patients by age group (considering children <2 years old vs. other patients) revealed considerable proportions of carriers of low-MBL genotypes among children <2 years old with IPD caused by opportunistic serotypes (46.2%), diagnosed with meningitis (42.9%), admitted to the ICU (40%) and of Caucasian ethnicity (36.8%). When comparing the frequency of low-MBL genotypes in these younger children and in other patients, ratios of proportions (R) between the two age groups were found to be statistically significant in Caucasian patients ($p = 0.013$) and in patients with IPD caused by opportunistic serotypes ($p = 0.020$). These results are recorded in Table 4.

TABLE 1. MBL2 genotype frequencies in 147 patients with invasive pneumococcal disease

MBL genotype group	Frequencies	%
YA/YA	42	28.6
YA/XA	37	25.2
XA/XA	6	4.1
Overall A/A	85	
YA/YB	21	14.3
XA/YB	6	4.1
YA/YC	7	4.8
XA/YC	2	1.4
YA/YD	11	7.5
XA/YD	2	1.4
Overall A/O	49	
YB/YB	4	2.6
YB/YC	3	2.0
YB/YD	4	2.6
YC/YC	1	0.7
YC/YD	0	0
YD/YD	1	0.7
Overall O/O	13	

Discussion

Susceptibility to IPD has been reported to be clearly related to age, and younger children are the group with the highest burden of disease [1,3]. The importance of the innate immune response in combating infections is well documented. Contrary to adaptive immunity, which takes days to generate and expand a specific humoral and/or cellular response against the pathogen, the innate response acts immediately (within minutes or hours). This innate immune system acts as a first-line defensive barrier, which is critical to contain the passage of nasopharyngeal colonizers to normally sterile sites until lymphocytes and specific antibodies take action [21]. It is also well known that the type and magnitude of the adaptive immune response vary with age, developing from immaturity at birth to maturity after the first 2 years of life, although it takes even longer for the adaptive immune system to fully develop. Therefore, in younger children the fight against infections mainly relies on the innate immune system.

An international study has analysed the association between mortality, clinical manifestations and recovery of invasive serotypes vs. non-invasive serotypes, and has shown that host factors are better predictors of associated morbidity and mortality of IPD than serotype invasiveness [8]. That is, whether an opportunistic serotype will cause disease and/or determine a worse evolution is more strongly related to a deficient host immunological response than to microbiological factors.

In the present study, we found a significantly high proportion of genotypes associated with low MBL production among children younger than 2 years with IPD. Moreover, the frequency of low-MBL genotypes was observed to be especially high (46.2%) in younger children with IPD caused by opportunistic serotypes. These data suggest that pneumococcal nasopharyngeal colonizers have more opportunities to cause invasive disease in young children with a genetically-determined low MBL production, which is a crucial factor in the innate immune response. Interestingly, when rates of low-MBL genotypes among patients with IPD caused by serotypes with high invasiveness (high-attack-rate serotypes) were analyzed, we did not find a significantly high proportion of low-MBL genotypes either in young children aged <2 years or in other patients. Serotypes with a high attack rate are bad colonizers, and different studies have shown that they have important virulence factors associated with the production of pleuropneumonia or other clinical manifestations of IPD [22]. These data suggest that in the case of high-attack-rate serotypes, microbiological factors may proportionally have

TABLE 2. Demographic, clinical, microbiological and genotypical characteristics of patients according to age group

Characteristics	<2 years	≥2 and <5 years	≥5 and <65 years	≥65 years	Total
Subjects	29 (19.7)	45 (30.6)	56 (38.1)	17 (11.6)	147
Sex					
Male	14 (48.3)	24 (53.3)	38 (67.9)	9 (52.9)	85 (57.8)
Female	15 (51.7)	21 (46.7)	18 (32.1)	8 (47.1)	62 (42.2)
Ethnicity					
Caucasian	19 (65.5)	31 (68.9)	45 (80.4)	15 (88.2)	110 (74.8)
Non-Caucasian	10 (34.5)	14 (31.1)	11 (19.6)	2 (11.8)	37 (15.2)
Clinical manifestation					
Pneumonia	18 (62.0)	39 (86.7)	53 (94.6)	15 (88.2)	125 (85.0)
Meningitis	7 (24.1)	2 (4.4)	1 (1.8)	2 (11.8)	12 (8.2)
Bacteraemia/sepsis	4 (13.8)	4 (8.9)	2 (3.6)	0	10 (6.8)
ICU admission (n = 137)[a]					
No	18 (64.3)	34 (79.1)	45 (90.0)	15 (93.8)	112 (83.0)
Yes	10 (35.7)	9 (20.9)	5 (10.0)	1 (6.3)	25 (17.0)
Clinical course (n = 129)[a]					
No sequelae	15 (62.5)	33 (86.8)	48 (96.0)	15 (88.2)	111
Sequelae	9 (37.5)	5 (13.2)	1 (2.0)	0	15
Exitus	0	0	1 (2.0)	2 (11.8)	3
IPD risk factor (n = 127)[a]					
No	23 (95.8)	38 (100.0)	39 (81.3)	5 (29.4)	105
Yes	1 (4.2)	0	9 (18.8)	12 (70.6)	22
Vaccine receipt					
No	21 (72.4)	26 (57.8)	50 (89.3)	17 (100)	114
PCV7	0	16 (35.6)	6 (10.7)	0	22
PCV10	1 (3.4)	1 (2.2)	0	0	2
PCV13	7 (24.1)	1 (2.2)	0	0	8
PCV7 & PCV13	0	1 (2.2)	0	0	1
Serotype invasiveness					
High	16 (55.2)	17 (37.8)	31 (55.4)	2 (11.8)	66 (44.9)
Non-high	13 (44.8)	28 (62.2)	25 (44.6)	15 (88.2)	81 (55.1)
PCV13 serotype					
No	7 (24.1)	13 (28.9)	19 (33.9)	13 (76.5)	52
Yes	22 (75.9)	32 (71.1)	37 (66.1)	4 (23.5)	95
MBL levels					
High (A/A)	10 (34.5)	22 (48.9)	36 (64.3)	11 (64.7)	79 (53.7)
Medium (XA/XA or A/O)	10 (34.5)	19 (42.2)	12 (21.4)	4 (23.5)	45 (30.7)
Low (X/A or O/O)	9 (31.0)	4 (8.9)	8 (14.3)	2 (11.8)	23 (15.6)
LOS (days)	14 (9.3–17.8)	9 (7.0–11.0)	8.5 (5.0–12.3)	7 (5.0–9.0)	9 (6.0–14.0)
ICU LOS days	2.5 (1.0–18.0)	1.0 (1.0–2.5)	1.0 (1.0–8.0)	0	1.0 (1.0–6.5)

Data are presented as n (%) or median (IQR).
[a]Missing values.
IPD, invasive pneumococcal disease; LOS, length of stay; ICU, intensive care unit; MBL, mannose-binding lectin; PCV7/PCV10/PCV13, 7-valent/10-valent/13-valent pneumococcal vaccine.

TABLE 3. Variable frequencies according to MBL level

Variables	Patients with low MBL level	Patients with medium/high MBL level	p-Value	Corrected p-value
Age				
<2 years old	**9 (31.0)**	20 (69.0)	**0.031**	0.186
≥2 years old	14 (11.9)	104 (88.1)		
Sex				
Male	14 (16.5)	71 (83.5)	0.747	1.000
Female	9 (14.5)	53 (85.5)		
Ethnicity				
Caucasian	16 (14.5)	94 (85.5)	0.526	1.000
Non-Caucasian	7 (18.9)	30 (81.1)		
Clinical manifestation				
Meningitis	4 (33.4)	8 (66.7)	0.191	0.955
Pneumonia/bacteraemia/sepsis	19 (14.1)	116 (85.9)		
ICU admission (n = 137)[a]				
No	18 (16.1)	94 (83.9)	0.826	1.000
Yes	5 (20.0)	20 (80.0)		
Serotype invasiveness				
High	8 (12.1)	58 (87.9)	0.290	1.000
Non-high	15 (18.5)	6 (81.5)		

Data are presented as n (%).
Significant values in bold numbers.
[a]Missing values.
ICU, intensive care unit; PCV13, 13-valent pneumococcal vaccine.

more weight in causing disease, regardless of the quality of the immune response.

Our data about the implication of MBL in the development of IPD in young children are novel but in agreement with studies performed in mice about the role of MBL in susceptibility to pneumococcal infection [23]. In addition, data also exist documenting the implication of MBL deficiency in other paediatric infectious diseases. A recent systematic review has

TABLE 4. Variable frequencies according to age group and MBL level

Variables	Age group <2 years (n = 29)		Age group ≥2 years (n = 118)		Patients with low MBL level	
	Total number of patients	Patients with low MBL level	Total number of patients	Patients with low MBL level	p-Value	R (95% CI)
Ethnicity						
Caucasian	19	7 (36.8)	91	9 (9.9)	**0.013**	**3.73 (1.58–8.76)**
Non-Caucasian	10	2 (20.0)	27	5 (18.5)	1.000	1.08 (0.25–4.70)
Clinical manifestation						
Meningitis	7	3 (42.9)	5	1 (20.0)	0.849	2.14 (0.30–15.07)
Others	22	6 (27.3)	113	13 (11.5)	0.121	2.37 (1.01–5.56)
ICU admission[a]						
No	18	5 (27.8)	94	13 (13.8)	0.263	2.01 (0.82–4.94)
Yes	10	4 (40.0)	15	1 (6.7)	0.128	6.00 (0.78–46.14)
Serotype invasiveness						
High	16	3 (18.8)	50	5 (10.0)	0.593	1.88 (0.50–6.99)
Non-high	13	6 (46.2)	68	9 (13.2)	**0.020**	**3.49 (1.50–8.12)**

Significant values in bold numbers.
[a]Missing values.
R, ratio of proportions; CI, confidence interval; ICU, intensive care unit; PCV13, 13-valent pneumococcal vaccine.

reported a probable association between HIV disease progression and MBL deficiency, and this association was especially high in children <2 years of age [24]. Dommett *et al.* [25] have reported the influence of MBL in the frequency and duration of infectious complications in children with malignancy. Finally, Koch *et al.* [26] report a statistical association of MBL insufficiency with the increase of risk of acute respiratory infection in children between 6 and 17 months. These results contrast with those documented in other populations without infectious diseases. A study performed in a cohort of newborns in the Netherlands showed that a low-MBL genotype was only observed in eight of 56 (14.2%) premature newborns and in two of 11 (18.1%) preterm neonates [27]. Another study performed in our geographical area reported a similar percentage of 15.3% among adults [13].

It was also of interest to find out that younger patients with pneumococcal meningitis showed a high proportion of low-MBL genotypes (42.9%), even though this proportion did not reach statistical significance when compared with the proportion of low-MBL genotypes in older patients. These data are in agreement with a recent study that reports an association between defective MBL genotypes and an increased risk of pneumococcal meningitis [28]. The hypothesis that MBL deficiency could be related to a worse clinical evolution should not be ruled out and deserves further analysis.

The significance of MBL binding *S. pneumoniae* is controversial because studies show low MBL binding to *S. pneumoniae* as well as to other encapsulated bacteria [29]. Therefore, other mechanisms distinct from complement-mediated opsonophagocytosis and bacterial killing of *S. pneumoniae* by MBL could be the basis of the clinical association reported here. In this regard, there is also evidence of direct interaction of MBL with phagocytic cells to promote phagocytosis and modify cellular activation [30], as well as increasing evidence in support of an immunomodulatory effect of MBL [31].

Our study should be interpreted in light of several limitations. First, although the study sample allowed us to obtain statistically significant results, analysis of more extensive populations should be undertaken to confirm our results, particularly in relation to the association between a genetically-determined MBL deficiency and the onset of pneumococcal meningitis. Second, our study does not exclude the putative contribution of other soluble pattern recognition innate immune proteins also potentially involved in the defence of the lungs against *S. pneumoniae* or other bacteria, such as surfactant proteins (SP-A and SP-D) [32], ficolins [33], pentraxins [34] or agglutinin gp-340/DMBT1 [35]. Third, we did not analyse other factors that may be involved in the step from colonization to disease and, in particular, the role of co-infection with respiratory viruses. Viral infection is very common in young children and it has been suggested that the acquisition of a virus damages the epithelial mucosa and promotes the expression of virulence determinants in the pathogen, which are related to adhesiveness to the mucosa and bacterial replication [36]. Moreover, the role of MBL in direct viral neutralization and inhibition of viral spread is well known [37]. Additional analysis is needed to determine whether deficiencies in innate immunity and co-infection with respiratory viruses together create the perfect situation for development of IPD in children.

In conclusion, our findings suggest an association of genetically determined low MBL production and IPD in younger children. Further confirmation of this novel association may open a pathway to the practice of personalized medicine in which paediatricians would not only evaluate the risk of IPD according to clinical, epidemiological and microbiological characteristics of the episode, but also according to predictive factors derived from the immune characteristics of the host. Our results also support the need to adopt a more integrated approach to the diagnosis and treatment of IPD in

young children in order to achieve better clinical outcomes for this particular group of patients. The challenge of finding a vaccine based on preserved pneumococcal proteins protecting against all serotypes could be the next response in the prevention of pneumococcal disease.

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Transparency Declaration

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