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ARTICLE

Estimation of the invasive disease potential of *Streptococcus pneumoniae* in children by the use of direct capsular typing in clinical specimens

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Abstract Traditionally, invasiveness indexes have been based on culture methods. We aimed to establish a new classification of the invasive disease potential of pneumococcal serotypes causing invasive pediatric disease in the era of conjugate vaccines in Catalonia, Spain, by adding capsular typing of *Streptococcus pneumoniae* in direct sample. Two samples of children attended at the University Hospital Sant Joan de Déu (Barcelona, Spain) between 2007 and 2011 were compared: a first sample of 358 children with invasive pneumococcal disease and a second sample of 402 pneumococcal nasopharyngeal carriers selected from 714 healthy children admitted for minor surgical procedures. The most common invasive serotypes were 1 (20.1 %, *n*=72), 19A (13.9 %, *n*=50), 3 (12.3 %, *n*=44), and 7FA (7.5 %, *n*=27), whereas the most common serotypes in carriage were 19A (8.7 %, *n*=38), 10FC33C (7.8 %, *n*=34), 6C (6.9 %, *n*=30), and 19FBC (5.5 %, *n*=24). We detected a rate of cocolonization of 26.4 % (*n*=89) among the 336 samples serotyped in the carriers population. Serotypes 1, 3, and 7FA were significantly associated with high invasiveness. Serotypes 6C, 10FC33C, 23A, 35B, 19FBC, 21, 11AD, 15BC, 23B, 34, and 6A were significantly associated with low invasiveness. Our results proved that the use of molecular techniques in direct sample for both the detection and the capsular identification of *Streptococcus pneumoniae* is very useful to obtain a more accurate calculation of the invasiveness of the different pneumococcal serotypes.

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Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide that especially affects young children and the elderly. The pneumococcus is normally found colonizing the mucosa of the human nasopharynx asymptomatically, sometimes even by more than one pneumococcal strain at the same time [1]. The World Health Organization (WHO) estimates that 476,000 deaths among children under 5 years old are caused by pneumococcal infections [2]. One of the main virulence factors of the pneumococcus is its polysaccharide capsule, from which more than 94 serotypes have been identified so far [3]. Despite their high diversity, only a minority of serotypes cause most of the invasive disease worldwide [4].

The invasive disease potential of a pneumococcal serotype shows its propensity to cause invasive disease. This parameter compares the frequency at which the serotype is found in invasive isolates with the frequency at which it is detected in healthy nasopharyngeal carriers [5, 6]. The estimation of the invasive disease potential of the different pneumococcal serotypes is an important tool for pediatricians and microbiologists to monitor which are the main serotypes with high risk to cause disease in children in their geographical area and presents data that can be useful for the development of future vaccines. Until now, this invasiveness index has been

traditionally based on culture methods. However, direct detection of pneumococcal DNA for diagnosis and capsular typing, which has proved to be more sensitive, may allow to establish a more accurate serotype distribution and potentially result in a change of the current invasive indexes. The addition of molecular techniques to culture could provide a more exact picture of the nasopharyngeal pneumococcal population based on their ability to detect low density and multiple-serotype carriage. Particularly for the invasive disease population, the use of these techniques may lead to a change in the real prevalence of some serotypes, such as 1 and 3, that could have been underestimated due to their difficult recovery by culture [7, 8].

Recently, our team has published a study where we estimated the invasiveness of pneumococcal serotypes by using the traditional methods based only on culture [9]. In the present study, the main goal is to determine the invasive disease potential of pneumococcal serotypes causing invasive pediatric disease in Catalonia, Spain, in the era of conjugate vaccines by adding capsular typing of *Streptococcus pneumoniae* in direct sample (without culture).

Materials and methods

Study design and sample collection

Two samples were compared. A first sample included all children with invasive pneumococcal disease (IPD) attended at the University Hospital Sant Joan de Déu (Barcelona, Spain) between 2007 and 2011. The second sample included healthy children who were identified as pneumococcal nasopharyngeal carriers by culture and/or specific DNA detection. Two healthy children for each IPD case were selected prospectively in wards and outpatient visits for non-infectious diseases and matched by age, sex, date of hospitalization or outpatient visit at the same center, and underlying risk condition when present. A specimen was obtained from each study participant. IPD was defined as the presence of clinical findings of infection, together with the isolation by culture and/or DNA detection by real-time polymerase chain reaction (PCR) of *S. pneumoniae* in any usually sterile fluid. In both samples, subjects were children up to 6 years old. Children were enrolled in the study if a parent or guardian provided written informed consent. The study was approved by the Sant Joan de Déu Ethical Committee of Clinical Research.

Microbiological identification

Pneumococcal strains were identified by standard microbiological methods that included the optochin sensitivity test and an antigenic test targeting the capsular polysaccharide (Slidex pneumo-kit, bioMérieux, Marcy-l'Etolie, France).

Molecular identification from direct sample

DNA detection in the invasive collection was performed by real-time PCR in normally sterile samples according to a published assay [10–12]. The presence of *S. pneumoniae* DNA was established by the amplification of the pneumolysin (*ply*) or autolysin (*lytA*) genes and the *wzg* (*cpsA*) gene. Only positive samples for the *ply*/*lytA* and *wzg* genes in real-time PCR were included in the study. The presence of the pneumococcus in the carriers collection was simply determined by the detection of the *lytA* and *wzg* genes. The DNA of *S. pneumoniae* was extracted using 20 % *w*/*v* Chelex 100 resin (Bio-Rad Laboratories, Hercules, CA). Two-hundred microliters of the medium of each nasopharyngeal swab were centrifuged at 4 °C for a period of 1 h. After centrifugation, the supernatant was discarded and 150 μl of the Chelex 100 resin was added to the pellet. Then, the mixture was briefly vortexed and incubated at 56 °C for 20 min, vortexed again, and incubated at 100 °C for 10 min. After cooling, the supernatant was used as the sample for the PCR techniques.

Serotype identification

Serotype identification from direct samples and from *S. pneumoniae* strains was performed at our laboratory using two different techniques. Serotype detection in the DNA obtained from direct sample with a real-time PCR-positive result above 30 cycles was performed by a fragment analysis technique based on fluorescent PCR reaction and subsequent fragment analysis by capillary electrophoresis. This technique was implemented in our laboratory in 2010. It is based on the use of 40 different primers pairs for the specific detection of 40 serotypes/serogroups and a pair of additional primers for detecting a fragment of the *cpsA* (*wzg*) gene, which is highly common to all capsular serotypes. So, this technique allows to detect all capsular types and to differentiate 40 serotypes/serogroups [13]. On the other hand, in samples with a positive result between 30 and 35 cycles, serotypes were identified by a published multiplex real-time PCR methodology that detects the *wzg* gene and differentiates 21 serotypes [12]. Such a technique identifies fewer serotypes but has been shown to be more sensitive than the previous method [14]. In addition, the serotyping of strains isolated by culture was completed by Quellung reaction at the National Center of Microbiology (Majadahonda, Spain).

Statistical analysis

The invasive disease potential of serotypes was estimated using odds ratios (ORs) with 95 % confidence intervals (95 % CIs), as described by Brueggemann et al. [5]. The equation used for the ORs calculations was $OR=(ad)/(bc)$, where a is the number of invasive X serotypes, b the number

of carriage X serotypes, *c* the number of invasive non-X serotypes, and *d* is the number of carriage non-X serotypes. An OR of 1 indicated that the serotype was equally likely to be recovered from invasive disease and carriage, whereas an OR >1 indicated an increased probability of causing invasive disease. The OR significance was tested with the two-tailed Fisher's exact test, using a cut-off p-value of ≤0.05 (two-tailed) for all statistical analyses. Also, the significance in the changes in serotypes during the study period and in the association between serotypes and multiple colonization was tested by the two-tailed Fisher's exact test, using the same cut-off p-value. The resulting p-values were corrected for multiple testing by controlling the false discovery rate (FDR) to ≤0.05 through the Benjamini and Hochberg method, as previously described [15, 16]. Simpson's index of diversity (SID) was used to measure the collections' diversity via the website http://www.comparingpartitions.info [17].

Results

Serotype distribution in disease and colonization

During the study period, 358 IPD cases were confirmed and serotyped (100 %), of which 240 (67 %) were only detected by PCR. We identified a total of 28 different serotypes. The most common ones were serotypes 1 (20.1 %, n=72), 19A (13.9 %, n=50), 3 (12.3 %, n=44), and 7FA (7.5 %, n=27). All of them are covered by the current pneumococcal conjugate vaccine PCV13. Overall, 7.3 % (n=26) of the serotypes were covered by PCV7, 38.5 % (n=138) by PCV10, and 66.5 % (n=238) by PCV13.

Out of the 714 healthy children enrolled as controls, 402 pneumococcal nasopharyngeal carriers were identified (colonization rate of 56.3 %); 183 (45.5 %) of them were only detected by PCR. We were able to serotype 336 of the 402 carriers (83.6 %), detecting a total amount of 434 serotypes related with multiple colonization. The samples that could not be serotyped presented a positive *lytA* PCR below 35 cycles. Overall, 41 different serotypes were identified. Among the total positive samples serotyped (n=434), the most common serotypes were 19A (8.7 %, n=38), 10FC33C (7.8 %, n=34), 6C (6.9 %, n=30), and 19FBC (5.5 %, n=24). Altogether, 14.7 % (n=64) of the serotypes were included in PCV7, 19.8 % (n=86) in PCV10, and 35.0 % (n=152) in PCV13.

The mean age of patients with IPD and carriers was 32 months [standard deviation (SD) 18.5] and 32 months (SD 17.7), respectively.

A PCV7 vaccine coverage rate of 36.0 % (95 % CI 31.23–41.13) was observed in children with IPD, compared to 49.2 % (95 % CI 44.50–54.12) in the carriers population (p < 0.01).

A comparison between the serotypes identified in both populations using SID revealed a higher diversity presence in the carrier population than in the invasive disease population: 0.953 (95 % CI 0.95–0.96) vs. 0.848 (95 % CI 0.83–0.87).

Serotype changes

We analyzed the changes of serotype distribution between the PCV7 era (2007–2009) and the PCV10-PCV13 era (2010–2011) (Figs. 1 and 2). Serotypes 1 and 7FA decreased from 24.3 to 13.2 % (p=0.01) and from 8.1 to 6.6 % (p=0.76), respectively. However, serotype 5 did not show any change between periods. Serotypes 3 and 6A increased from 10.8 to 14.7 % (p=0.35) and from 0.9 to 2.9 % (p=0.3), respectively, but no tendency was observed in serotype 19A. PCV7 serotypes continued to decrease during the study period.

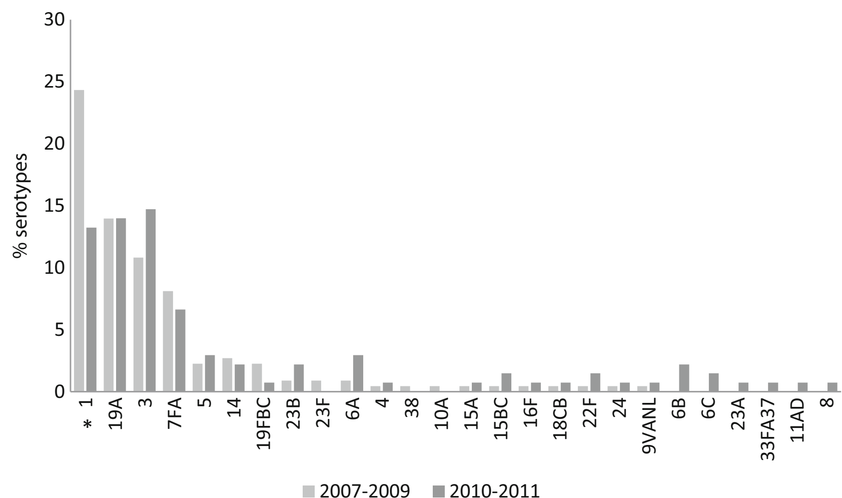

Fig. 1 Distribution of serotypes in invasive pneumococcal disease (IPD) from the PCV7 period (2007–2009) to the PCV10 period (2010–2011). *p < 0.05, #other serotypes presented a proportion of 26.6 % in invasive disease during 2007–2009 and 26.5 % during 2010–2011

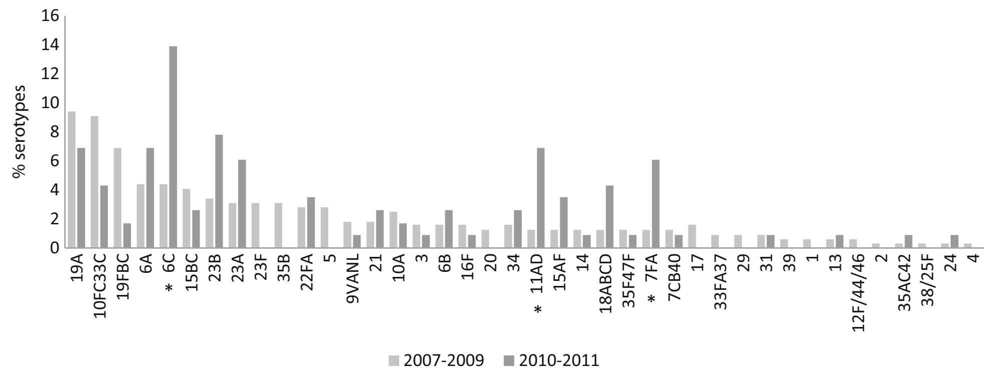

Fig. 2 Distribution of serotypes in nasopharyngeal carriers from the PCV7 period (2007–2009) to the PCV10 period (2010–2011). *$p < 0.05$, #other serotypes presented a proportion of 12.2 % in carriers during 2007–2009 and 6.9 % during 2010–2011

Detection of multiple colonization in carriage

We detected a rate of cocolonization of 26.4 % (n=89) among the 336 samples serotyped in the carriers population. Most of the cocolonization cases were produced by two serotypes (75.3 %, n=67), but in some samples, we were able to find the presence of three (16.8 %, n=15) and even four (7.9 %, n=7) different serotypes colonizing the children's nasopharynx. Some serotypes were more frequently found as simple colonizers, while others presented a clear tendency to cocolonize. Some significant associations were found between serotypes and type of colonization: with multiple colonization serotypes 10FC33C ($p < 0.01$) and 7CB40 (p=0.04), and with single colonization serotype 19A (p=0.05) (Fig. 3).

Serotype invasiveness

There were important differences in the invasive disease potential of the pneumococcal serotypes analyzed in this study (Table 1). Serotypes 1, 3, and 7FA, all included in PCV13, were associated with high invasiveness presenting and OR significantly higher than 1, even after the FDR correction was applied. On the other hand, serotypes 6C, 10FC33C, 23A, 35B, 19FBC, 21, 11AD, 15BC, 23B, 34, and 6A were associated with low invasiveness, showing a significantly OR lower than 1.

Discussion

The impact of conjugate vaccines has produced an important change in the pneumococcal serotypes circulating all over the world. In a previous study, we analyzed this impact in our geographical area using traditional methods based on culture [9]. In the present study, we aimed to add the molecular techniques for both the detection and the serotyping of the pneumococcus. It has already been proven that molecular techniques greatly improve the detection of pneumococcus, but, to date, most of the invasiveness studies published have utilized serotyping techniques based on culture [5, 16, 18–20]. Even these studies that adopt molecular techniques for the identification of the pneumococcal capsule usually require previous isolation of the strain [21]. In our study, we were interested in evaluating the changes produced in the classification of the invasive potential of pneumococcal serotypes as a result of the implementation of serotyping in direct sample.

Similarly to our previous results, serotype 1 appeared as the most common serotype in the invasive population, but the addition of the molecular techniques almost doubled the rate of detection in the present study, showing the real magnitude of this serotype in our geographical area. Underestimation of serotype 1 detection due to culture methods has recently been discussed to be the cause of the changing prevalence of such serotype in different geographical areas, due to the effects of antibiotic administration prior to sampling [7]. Serotypes 19A

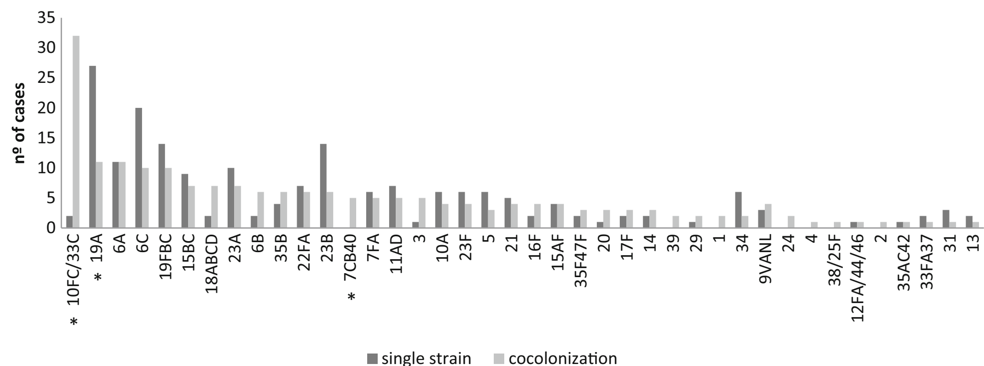

Fig. 3 Serotype distribution in nasopharyngeal carriers according to their detection as a single strain or in cocolonization (2007–2011). *$p < 0.05$

Table 1 Invasive disease potential of serotypes

Serotype	No. of isolates		OR	95 % CI	*p*-Value[a]
	Invasive	Carriage			
PCV7	26	64			
6B	3	8	0.45	0.12–1.70	0.486
14	9	5	2.21	0.73–6.66	0.371
19FBC	6	24	0.29	0.12–0.72	**0.029**
23F	2	10	0.24	0.05–1.09	0.174
4	2	1	2.43	0.22–26.93	0.946
9VANL	2	7	0.34	0.07–1.66	0.42
18ABCD	2	9	0.26	0.06–1.23	0.226
PCV10	138	86			
1	72	2	54.37	13.24–223.39	**<0.01**
5	13	9	1.78	0.75–4.21	0.4
7FA	27	11	3.14	1.53–6.41	**0.01**
PCV13	238	152			
3	44	6	9.99	4.20–23.74	**<0.01**
6A	6	22	0.32	0.13–0.80	**0.045**
19A	50	38	1.7	1.08–2.64	0.066
Non-PCV13	120	282			
6C	2	30	0.07	0.02–0.31	**<0.01**
10A	1	10	0.12	0.01–0.93	0.065
11AD	1	12	0.1	0.01–0.76	**0.033**
15AF	2	8	0.3	0.06–1.41	0.308
15BC	3	16	0.22	0.06–0.76	**0.04**
16F	2	6	0.4	0.08–1.99	0.547
17F	0	5	0	0.00–0.00	0.203
21	0	9	0	0.00–0.00	**0.032**
22FA	3	13	0.27	0.07–0.97	0.119
23A	1	17	0.07	0.01–0.52	**<0.01**
23B	5	20	0.29	0.10–0.79	**0.042**
8	1	0	0	0.00–0.00	0.973
38/25F	1	1	1.21	0.07–19.46	>0.99
24	2	2	1.21	0.17–8.66	>0.99
33FA37	1	3	0.4	0.04–3.88	0.878
13	0	3	0	0.00–0.00	0.459
10FC33C	0	34	0	0.00–0.00	**<0.01**
35F47F	0	5	0	0.00–0.00	0.194
39	0	2	0	0.00–0.00	0.739
20	0	4	0	0.00–0.00	0.313
35B	0	10	0	0.00–0.00	**0.024**
29	0	3	0	0.00–0.00	0.444
12FA/44/46	0	2	0	0.00–0.00	0.718
2	0	1	0	0.00–0.00	>0.99
34	0	8	0	0.00–0.00	**0.042**
31	0	4	0	0.00–0.00	0.3
35AC42	0	2	0	0.00–0.00	0.698
7CB40	0	5	0	0.00–0.00	0.185
Others	95	47			
Total	358	434			

[a] *p*-Values that were significant after false discovery rate (FDR) correction are shown in **bold** type

and 7FA also continued to be in the range of the most common serotypes causing invasive disease. The most interesting finding was the high detection of serotype 3. In our previous study, the results already suggested a high invasive disease potential of this serotype, but without reaching significance after FDR correction. In the present study, not only was serotype 3 significantly associated to a high invasive disease potential, but it was also shown to be the third most common serotype in children with invasive disease. Detection of this serotype represented a 5-fold increase with respect to detection by culture. It is noteworthy that, although several published studies have reported a lower frequency of serotype 3 in children [22, 23], a recent study in our geographical area discussed the fact that serotype 3 could have been underestimated in children due to difficulties in recovering it in culture [8].

As shown in other publications, temporal changes in serotypes were detected throughout the study period [23, 24]. We observed a reduction of serotypes 1 and 7FA, as well as an increase of serotypes 3 and 6A. Even though the introduction of conjugate vaccines may be associated with these changes, other factors could influence these dynamics. Recently, our group has reported a significant decrease of serotype 1 associated with the circulation of H1N1 pandemic flu virus [25].

In the carriers population, important changes were observed thanks to the ability of molecular techniques to detect multiple colonization. The capability to identify different serotypes present in the nasopharynx of one child at the same time greatly increased the number of serotypes that could be identified among carriers. These serotypes were most probably masked by other serotypes that are more predominant and more easily recovered by culture. One of the main effects of the improved detection of serotypes by molecular methods was that some serotypes that were poorly found or not found at all in carriers by culture increased their number, causing an adjustment of the invasive disease potential of these serotypes. In our previous results, serotype 5 was significantly associated to invasive disease, but it did not reach significance in the new analysis because a major number of episodes were identified in carriers. On the other hand, serotype 6A, which only showed a tendency to be more present in carriers, was significantly associated to carriage in the current study. The new analysis also revealed that more serotypes were associated to carriage. Although we had found that serotypes 19A and 19FBC were between the most common serotypes in carriers in our previous study, serotypes 10FC33C and 6C appeared as the ones more frequently found in the nasopharynx of healthy children in this study. Another interesting result was the detection of serotype 1 in carriers. This is a rare event even in areas where such serotype is the main cause of IPD, usually reported only during outbreaks of this serotype [26]. Some studies have suggested that the lack of serotype 1 detection in carriers could be due to the lack of techniques able to detect multiple colonization [27, 28]. In fact, in our study, both cases

of serotype 1 corresponded to cocolonization; however, Nunes et al. have suggested that this fact is a consequence of the effect of PCV7 [29].

The results showed a low rate of PCV7 serotypes in the nasopharynx of healthy children as it occurred in the invasive population. Surprisingly, serotype 19FBC seemed to persist, resulting in a significant association with carriage. However, in the analysis of the serotype changes, we observed a decrease during the study period. This different rate of disappearance of serotype 19FBC has been observed in other carriage studies [30, 31].

One potential limitation of our study is that our technique is not able to distinguish all the pneumococcal serotypes that have been identified to the present day. However, it distinguishes the main serotypes found to be causing invasive disease and covered by the conjugate vaccines, which are the ones of the main concern to public health.

In conclusion, the results of this study proved that the use of molecular techniques in direct sample for both the detection and the capsular identification of *S. pneumoniae* is very useful to obtain a more accurate distribution of the pneumococcal population in the invasive disease and in the nasopharynx of children in Spain, in addition to offering a closer approximation to reality of the invasiveness of the different pneumococcal serotypes.

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Conflict of interest The authors declare no conflicts of interest.

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