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ARTICLE

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

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

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Introduction

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

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

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

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

Materials and methods

Patients and setting

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

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

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

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

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

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

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

Molecular diagnosis of *S. pneumoniae*

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

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

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

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

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

Statistical analysis

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

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Differences in serotype distribution according to age and microbiological diagnosis technique

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

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

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

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

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

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

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

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

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

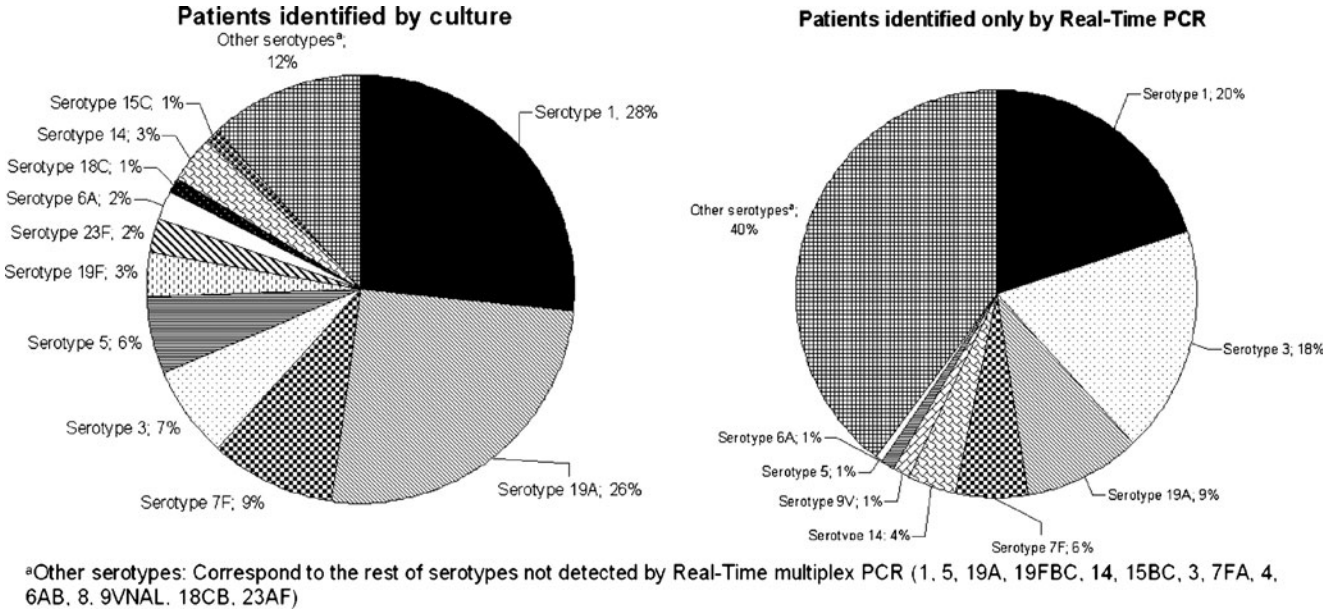

Fig. 1 Serotype distribution according to diagnosis by culture or only real-time polymerase chain reaction (PCR)

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

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

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

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

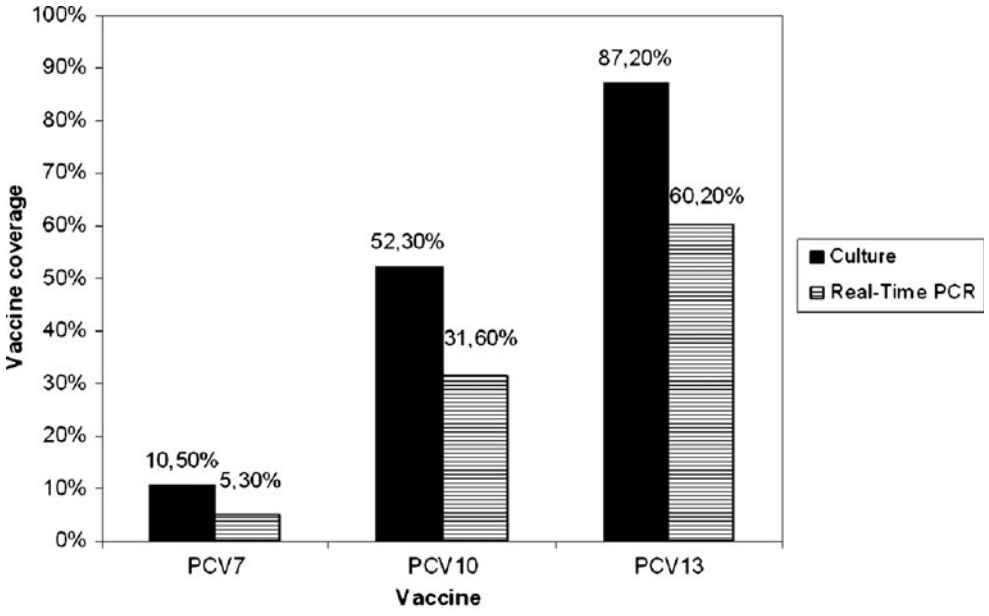

Fig. 2 Coverage of different pneumococcal conjugate vaccines (PCVs)

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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