Characterization of *Streptococcus pneumoniae* clones from paediatric patients with cystic fibrosis

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The role of *Streptococcus pneumoniae* in cystic fibrosis (CF) is poorly understood. The pneumococcal population has changed over time after the introduction of the heptavalent conjugate vaccine (PCV7) and, more recently, the 13-valent conjugate vaccine (PCV13). Although serotypes and clones causing invasive pneumococcal disease or colonizing healthy children have been extensively analysed, little is known so far on the serotypes and clones of pneumococci in CF patients. The aim of this work was to investigate serotypes, antibiotic susceptibilities, genotypes and biofilm production of CF pneumococcal isolates. Overall, 44 *S. pneumoniae* strains collected from 32 paediatric CF patients from January 2010 to May 2012 in a large Italian CF Centre were tested for antimicrobial susceptibility testing by Etest, serotyped by the Quellung reaction and genotyped by a combination of different molecular typing methods, including *pbp* gene restriction profiling, *pspA* restriction profiling and sequencing, PFGE and multilocus sequence typing. Biofilm production by pneumococcal strains was also assessed. Penicillin non-susceptibility was 16 %.

High resistance rates (>56 %) were observed for erythromycin, clindamycin and tetracycline. The most frequent serotype recovered was serotype 3 (31.8 %). The coverage of PCV7 and PCV13 was 6.8 and 47.7 %, respectively. More than 80 % of CF strains belonged to Pneumococcal Molecular Epidemiology Network (PMEN) reference clones, the most common being Netherlands3-ST180 (28.2 %), and Greece21-30/ST193 (15.4 %). All strains produced biofilm in vitro, although with large variability in biofilm formation efficiency. No correlation was found between biofilm levels and serotype, clone or antibiotic resistance. The high isolation rate of antibiotic-resistant serotype 3 pneumococci from CF patients suggests that PCV13 could increase protection from pneumococcal colonization and infection.

**INTRODUCTION**

Cystic fibrosis (CF) patients characteristically present chronic bacterial colonization of the broncho-pulmonary compartment without tissue invasion, followed by infectious exacerbations that lead to progressive deterioration of lung function (Cantón & del Campo, 2010). It has not been reported that CF patients represent a high-risk population in terms of contracting invasive bacterial diseases.
A few bacterial species are commonly involved in CF lung infections, although a number of other species with uncertain roles in disease progression may be recovered from the CF respiratory tract (LiPuma, 2010). The occurrence of co-colonization by various micro-organisms complicates the picture and probably participates in the hyperstimulation of the immune system, contributing to the evolution of CF disease (Cantón & del Campo, 2010).

Streptococcus pneumoniae is able to cause both non-invasive (i.e. otitis and pneumonia) and invasive [invasive pneumococcal disease (IPD)] infections (i.e. bacteremia and meningitis). The polysaccharide capsule represents one of the principal virulence factors of S. pneumoniae, determining >90 pneumococcal capsular serotypes, some of which are able to cause IPD (Hausdorff et al., 2000).

After the introduction of the heptavalent pneumococcal conjugate vaccine (PCV7), targeting the seven most common serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) causing IPD in infants and children, the rate of IPD due to vaccine serotypes (PCV7-VSs) decreased dramatically, whilst the rate due to serotypes not included in PCV7 (PCV7-non-vaccine serotypes, PCV7-NVSs) increased (Rosen et al., 2011). After the introduction of PCV7, a reduction in nasopharyngeal colonization due to PCV7-VSs was observed, although the overall rate of pneumococcal carriage generally remained unchanged, due to replacement of PCV7-VSs with PCV7-NVSs (Sharma et al., 2013). Moreover, vaccination with PCV7 caused a decrease in pneumococcal infections caused by penicillin- and multidrug-resistant isolates, which were commonly associated with VSs, with a concomitant small increase in infections due to antibiotic-resistant PCV7-NVSs (Kyaw et al., 2006). PCV7 has been available in Italy since 2001, reaching a mean national coverage in 2008 of 55%, although with wide local differences (ICONA Working Group, 2009). Since 2010, PCV7 has been replaced by the 13-valent pneumococcal conjugate vaccine (PCV13), containing six additional serotypes (1, 3, 5, 6A, 7F and 19A) (Alfonsi et al., 2011).

The pneumococcal surface protein A (PspA), encoded by the pspA gene, has been investigated as a vaccine candidate, as an alternative to polysaccharide-conjugated vaccines (Ogunniyi et al., 2007). The pspA sequence from pneumococcal strains in different countries provides information on the major PspA types circulating, which is useful for vaccine development (Rolo et al., 2009). The pspA gene has also been used as an epidemiological typing marker in pneumococci (Beall et al., 2000; Gherardi et al., 2012).

β-Lactam antibiotics are the most important drugs in the treatment of pneumococcal infections. β-Lactam resistance is associated with alterations in pbp genes, mainly pbp1a, pbp2b and pbp2x, encoding penicillin-binding proteins (PBP), which act as transpeptidases in peptidoglycan biosynthesis (Goffin & Ghysen, 2002). Consequently, profiles of pbp genes have been used successfully in predicting penicillin susceptibility (Gherardi et al., 2012).

Biofilm formation is a hallmark of chronic infections, including CF ones where the biofilm is typically polymicrobial (Peters et al., 2012). In these clinical manifestations, biofilms seem to contribute to bacterial persistence due to resistance to the host immune response and antibiotic treatment (Donlan & Costerton, 2002). Biofilm formation, although typical of some CF pathogens such as Pseudomonas aeruginosa and Staphylococcus aureus, has also been reported for pneumococci (Oggioni et al., 2006; Camilli et al., 2011).

Although serotypes and clones causing IPD or colonizing healthy children have been analysed extensively, very few studies have included CF patients (del Campo et al., 2005; Maeda et al., 2011), probably because this bacterial species is rarely isolated from CF patients. The aim of this work was therefore to investigate serotypes, antibiotic susceptibilities, genotypes and the biofilm-forming ability of S. pneumoniae isolates from the respiratory tract of CF children, a population where selective pressure imposed by vaccination and frequent antibiotic use is high.

METHODS

Patients and clinical data. The CF Centre of the Paediatric Hospital ‘Bambino Gesù’ in Rome is a large centre attended by approximately 300 CF patients year−1. All patients seen at the centre during the period January 2010 to May 2012 were included in the study. According to the protocol of the centre, patients are submitted annually to at least four surveillance cultures of respiratory secretions obtained by tracheobronchial aspirates. All CF patients received pneumococcal vaccination. Demographic characteristics and clinical data of patients with at least one S. pneumoniae isolation from a tracheobronchial aspirate were obtained by reviewing their medical charts. No healthy controls were included in this study.

The study was approved by the Ethical Committee of the Paediatric Hospital Bambino Gesù. Informed consent was obtained from parents or guardians.

Sample collection and microbiological procedures of isolation and identification. Due to different compliance of the patients or to their clinical conditions, the number of respiratory specimens obtained from each patient during the study period ranged between 1 and 21, with a mean of six specimens per patient. Respiratory pathogens from CF patients were detected and identified using conventional culture and identification methods (Bittar & Rolain, 2010). In addition, bacterial species were identified by matrix-assisted laser system desorption-ionization time-of-flight mass spectrometry using a Bruker Biotype software 2.0 (Bruker Daltonics). Partial 16S rRNA gene amplification and sequencing was used when necessary, especially for non-fermenting Gram-negative bacterial species (Ferroni et al., 2002). Pneumococcal isolates were identified on the basis of colony morphology, z-haemolysis on blood agar, an optochin test and a bile solubility test (Spellerberg & Brandt, 2011). z-Haemolytic streptococci with colonies of mucoid appearance or with a central navel-like depression that were optochin susceptible and bile soluble were identified as S. pneumoniae. Isolates were stored in the Cryobank (Mast Group) at −80°C until use.

Antimicrobial susceptibility testing and serotyping of pneumococci. Antimicrobial susceptibility testing was performed by Etest (bioMérieux) using the breakpoints recommended by the European
Table 1. Demographic data of CF patients carrying *S. pneumoniae*, co-colonizing organisms and characteristics of the pneumococcal isolates (serotypes, antimicrobial resistance and PFGE types)

| Patient (gender)* | Age (years) | Co-colonizing organisms† | Isolate ID‡ | Date of isolation (month/year) | PFGE type§ | Serotype | Antimicrobial non-susceptibility phenotype|| | MIC penicillin (mg l⁻¹) |
|-------------------|-------------|--------------------------|-------------|-------------------------------|------------|----------|--------------------------------|-----------------|------------------------|
| 1 (F) 2           | 2           | HI, NFGN                 | PFC01       | 01/10                         | 12         | 3        |                                 |                 | ≤0.03                  |
| 2 (M) 10          | 2           | MC, SA, NFGN             | PFC02       | 02/10                         | 41         | 19A      | ERY, CLI, TET                   |                 | ≤0.03                  |
| 3 (M) 5           | 10          | GP                       | PFC03       | 02/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 4 (M) 10          | 5           | NFGN                     | PFC04       | 02/10                         | 39         | 23B      | ERY, CLI, TET                   | 0.06            |
| 5 (F) 6           | 10          | HI, MC, PA, SA, SM       | PFC16       | 03/10                         | 93         | 16       | ERY, CLI, TET                   | 0.06            |
| 6 (F) 7           | 2           | M                        | PFC17       | 04/11                         | 59         | 23A      | PEN                            | 0.25            |
| 7 (F) 5           | 2           | SA                       | PFC18       | 03/10                         | 37         | 15C      | ERY, CLI, TET                   | 0.03            |
| 8 (M) 2           | 2           | MC, SA, EC               | PFC19       | 02/10                         | 37         | 15C      | ERY, CLI, TET                   | 0.03            |
| 9 (M) 4           | 2           | HI, SA                   | PFC20       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.06            |
| 10 (M) 5          | 2           | HI, PA                   | PFC21       | 11/10                         | 30         | 22F      |                                 | 0.03            |
| 11 (F) 2          | 2           | SA                       | PFC22       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 12 (F) 4          | 2           | SA                       | PFC23       | 03/10                         | 37         | 15B      | PEN, ERY, CLI, TET              | 0.25            |
| 13 (F) 6          | 2           | SA                       | PFC24       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.06            |
| 14 (M) 3          | 2           | SA                       | PFC25       | 03/10                         | 55         | 11A      | ERY                            | 0.03            |
| 15 (M) 4          | 2           | SA                       | PFC26       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 16 (M) 10         | 2           | HI, SA                   | PFC27       | 03/10                         | 95         | 6C       | PEN                            | 0.25            |
| 17 (M) 4          | 2           | HI, MC, PA               | PFC28       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.06            |
| 18 (M) 16         | 2           | None                     | PFC29       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 19 (F) 5          | 2           | SA, EC                   | PFC30       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 20 (F) 1          | 2           | SA                       | PFC31       | 03/10                         | 96         | 6A       | PEN, ERY, CLI, TET              | 0.03            |
| 21 (M) 3          | 2           | SA                       | PFC32       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 22 (F) 11         | 2           | SA, Y                    | PFC33       | 03/10                         | 4          | 6C       | PEN, ERY, CLI, TET              | 0.125           |
| 23 (F) 7          | 2           | HI                       | PFC34       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 24 (F) 1          | 2           | HI, PA, SA, SM           | PFC35       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 25 (M) 4          | 2           | HI, SA, GP               | PFC36       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.06            |
| 26 (M) 4          | 2           | HI, NFGN                 | PFC37       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.03            |
| 27 (M) 4          | 2           | HI                       | PFC38       | 03/10                         | 12         | 3        | ERY, CLI, TET                   | 0.06            |

*Gender: F = female, M = male.
†Co-colonizing organisms include HI = haemolytic streptococcus, SA = Staphylococcus aureus, NFGN = normal flora, GP = Gram positive, MC = methicillin resistant Staphylococcus aureus, PA = Pseudomonas aeruginosa, SM = Staphylococcus epidermidis, EC = Enterococcus, MY = Mycobacterium abscessus.
‡Isolate ID is a unique identifier for each isolate.
§PFGE type is a genetic fingerprint used to identify the genetic similarity of the isolates.
|| denotes various combinations of antibiotics to which the isolate is non-susceptible.
Table 1. cont.

<table>
<thead>
<tr>
<th>Patient (gender)*</th>
<th>Age (years)</th>
<th>Co-colonizing organisms</th>
<th>PFGE type</th>
<th>Scortype</th>
<th>Antimicrobial non-susceptibility to (mg l⁻¹)</th>
<th>MIC penicillin</th>
<th>Date of isolation (month/year)</th>
<th>Isolate ID</th>
<th>Co-colonizing organisms</th>
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<td>PFC71</td>
<td>D</td>
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<td>05/12</td>
<td>PFC71</td>
<td>HI, SM</td>
</tr>
<tr>
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<td>HI</td>
<td>PFC59</td>
<td>D</td>
<td>PEN, CTX, ERY, CLI, TET</td>
<td>0.06</td>
<td>05/12</td>
<td>PFC59</td>
<td>HI, SM</td>
</tr>
<tr>
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<td>HI, MC, PA</td>
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<td>D</td>
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<td>03/12</td>
<td>PFC63</td>
<td>HI, MC, PA</td>
</tr>
<tr>
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<td>23F</td>
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<td>03/12</td>
<td>PFC68</td>
<td>HI, MC, PA</td>
</tr>
<tr>
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<td>PFC69</td>
<td>D</td>
<td>PEN, CTX, ERY, CLI, TET</td>
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<td>03/12</td>
<td>PFC69</td>
<td>HI, MC, PA</td>
</tr>
<tr>
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<td>D</td>
<td>PEN, CTX, ERY, CLI, TET</td>
<td>0.03</td>
<td>04/12</td>
<td>PFC70</td>
<td>HI, MC, PA</td>
</tr>
</tbody>
</table>

*Female, M, male. +EC, Escherichia coli; HI, Haemophilus influenzae; MC, Moraxella catarrhalis; PA, Pseudomonas aeruginosa; SA, Staphylococcus aureus; SM, Stenotrophomonas maltophilia; SP, Staphylococcus pneumoniae; NFGN, non-fermentative Gram-negative (spp. other than P. aeruginosa: Achromobacter xylooxidans and Bordetella bronchiseptica); Y, yeast (Candida albicans); M, mould (Aspergillus).

Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org/clinical-breakpoints/). Isolates were tested for susceptibility to penicillin, cefotaxime, erythromycin, clindamycin, tetracycline and levofloxacin. As EUCAST breakpoints for penicillin (non-meninitis) indicate susceptibility at a MIC of <0.12 mg l⁻¹ and resistance at a MIC of ≥2 mg l⁻¹, isolates showing a MIC of ≥0.12 mg l⁻¹ were considered to be penicillin non-susceptible S. pneumoniae (PNSSP). An isolate was defined multidrug resistant if it was resistant to at least three antibiotic classes among those tested. Serotyping was performed by the Quellung reaction using antisera from the Statens Serum Institut, as described previously (Spellerberg & Brandt, 2011).

### Analysis of pbp2b, pbp2x and pspA genes

Restriction profiles of the PBP genes pbp2b and pbp2x were determined as described previously (Gherardi et al., 2000). To further characterize CF strains, the pspA gene was amplified and digested with the restriction enzyme DdeI and/or sequenced as described previously (Beall et al., 2000). By pspA gene sequence-based analysis, PspA proteins can be classified into three families, which are further subdivided into six clades: family 1 with clades 1 and 2; family 2 with clades 3, 4 and 5; and family 3 with clade 6 (Beall et al., 2000).

### PFGE and multilocus sequence typing (MLST)

S. pneumoniae isolates were examined by PFGE following Smal macrorestriction, as described previously (Gherardi et al., 2000). Isolates with profiles differing by one to six bands were assigned to the same PFGE type, whilst isolates with more than six band differences were assigned to different PFGE types. PFGE profiles were compared with those found in previous studies and received the same type designation (Dicuonzo et al., 2002; Gherardi et al., 2009, 2012). PFGE profiles were also analysed with BioNumerics software for Windows (version 2.5; Applied Maths). Comparison was performed using the unweighted pair group method with arithmetic averages (UPGMA) and with the Dice similarity coefficient, applying a 1.5% tolerance in band position. PFGE profiles with >80% similarity belonged to the same PFGE type (Beall et al., 2006). The two methods used to compare and interpret PFGE profiles – visual comparison and computer-assisted analysis – were totally consistent in determining the genetic relatedness of pneumococcal strains used in this study.

The clone corresponding to each PFGE type was inferred by the previously demonstrated association between each PFGE type and a specific international clone as defined by the Pneumococcal Molecular Epidemiology Network (PMEN; http://web1.sph.emory.edu/PMEN/) and/or a specific sequence type (ST) obtained by MLST (Dicuonzo et al., 2002; Gherardi et al., 2009, 2012). All PFGE types represented by more than one isolate were assigned to a corresponding ST (Gherardi et al., 2012).

### Biofilm production assay

Biofilm formation was assessed as described previously (Camilli et al., 2011) with minor modifications. Six out of 39 non-duplicate isolates could not be tested for biofilm formation because they were no longer viable at the time of the experiment. Briefly, pneumococci were grown in Todd–Hewitt broth at 37 °C for 18 h in the presence of 5% CO₂, diluted in the same medium supplemented with 1% glucose and dispensed into 96-well polystyrene microtitre plates (Nunc As). After 18 h of incubation, the biofilm samples were washed three times with PBS, air dried for 2 h and stained with Hucker’s crystal violet. The excess stain was removed by four washes with water, and the remaining stain was solubilized by adding 95% ethanol for 20 min at −20 °C. Biofilm biomass was evaluated by measuring the absorbance at 570 nm (A₅₇₀) of the eluate. The low cut-off was calculated as the mean A₅₇₀ ± 3 SD of uninoculated control wells. Biofilm levels were normalized by the bacterial growth, assessed spectrophotometrically at 570 nm. The biofilm index was calculated as A₅₇₀/₅₇₀. Differences in biofilm index
were analysed by an unpaired t-test or Mann–Whitney test (between groups, for parametric and non-parametric results, respectively) or by analysis of variance or Tukey or Kruskall–Wallis test followed by Dunn's multiple comparison post-test (among groups, for parametric and non-parametric results, respectively), considering $p<0.05$ as statistically significant. *Acinetobacter baumannii* ACICU strain (Antunes et al., 2011) was included as a positive control for biofilm production.

**RESULTS**

**Epidemiological background**

A total of 300 CF patients attending the ‘Bambino Gesù’ Paediatric Hospital in Rome were included in an approximately 2.5-year follow-up study. Thirty-two patients had at least one respiratory sample positive for *S. pneumoniae*, giving a total of 44 positive samples. Twenty-three patients (71.9%) had a single positive sample, whilst nine patients (28.1%) had from two to four cultures testing positive for *S. pneumoniae* at different times (Table 1). The yearly incidence of *S. pneumoniae* isolation was 4.8% in 2010 (14 samples positive out of 292 samples analysed), 7.4% in 2011 (22 out of 298) and 6.9% during the first 5 months of 2012 (8 out of 116). The age of patients ranged from <1 to 16 years (median: 4.5 years) (Table 1). All but one patient had been vaccinated with PCV7 and one patient (the oldest) with the 23-valent polysaccharide vaccine. A chart review revealed that, at the time of *S. pneumoniae* isolation, none of the patients presented with clinical or laboratory evidence of pulmonary exacerbation. Therefore, *S. pneumoniae* could be considered a colonizer of the respiratory tract, designating its recovery as ‘colonization episodes’.

In 24 episodes (55%), patients had received one or more antibiotics in the previous month: an aminoglycoside (12 episodes, 27.3%), a macrolide (11 episodes, 25%), a fluoroquinolone (six episodes, 13.7%) or a $\beta$-lactam (four episodes, 9.1%).

**Microbiological data**

Bacterial cultures of the respiratory samples revealed that *S. pneumoniae* was recovered in association with other respiratory pathogens in 37 out of 44 episodes (84.1%). Bacterial counts of *S. pneumoniae* and other respiratory colonizers/pathogens ranged between $10^5$ and $10^6$ c.f.u. ml$^{-1}$. *Haemophilus influenzae* was the most commonly isolated species in association with *S. pneumoniae* (19 out of 44 episodes, 43.2%), followed by *Staph. aureus* (15 out of 44 episodes, 38.6%), *Moraxella catarrhalis* (7 out of 44, 15.9%), *P. aeruginosa* and *Escherichia coli* (five out of 44 episodes, 11.4% each).

**Antibiotic resistance and serotypes**

The antimicrobial resistance profiles and serotypes of the pneumococcal isolates are shown in Table 1. Penicillin non-susceptibility was found in seven out of 44 isolates (15.9%), with six isolates showing a MIC for penicillin of between 0.125 and 1 mg l$^{-1}$ and only one isolate being fully penicillin resistant, with a MIC equal to 4 mg l$^{-1}$ (Table 1). Only three PNSSP isolates were cefotaxime non-susceptible. High resistance rates were observed for erythromycin, clindamycin and tetracycline (61.4, 56.8 and 59.1%, respectively), whilst all isolates were fluoroquinolone susceptible (Table 1).

Among the 44 isolates, 18 different serotypes were observed. The predominant serotype was serotype 3 (14 isolates, 31.8%), followed by serotypes 6C, 15B, 15C, 23A and 23B (three isolates each, 6.8%). Only 6.8% of the isolates were PCV7-VSSs, whilst 47.7% of the isolates were PCV13-VSSs. Among the seven PNSSPs, only one belonged to PCV7-VS (serotype 14), whilst the remaining six were PCV13-NVS (Table 1).

**Molecular analysis and clonality**

Overall, 18 different PFGE types were found among the 44 isolates. As shown in the dendrogram, the PFGE profiles of isolates belonging to the same PFGE type displayed >80% similarity (Fig. 1). Thirteen PFGE types, accounting for 39 of the 44 strains (88.6%), corresponded to a PMEN clone or to a clonal complex (CC) present in the MLST database (Table 2, Fig. 1).

Serotyping and molecular analysis allowed the detection of multiple *S. pneumoniae* colonization episodes in nine patients. In four patients, a pneumococcal strain of the same serotype, antibiotic susceptibility pattern and PFGE type was detected over time. In five patients, isolates of different serotype and genotype were found, suggesting repeated colonization (Table 1).

Only one isolate with similar characteristics per child was included in the clonal analysis. Out of 39 non-duplicate isolates, 32 showed PFGE types typically associated with a PMEN clone (Table 2, Fig. 1). Four clones were represented by isolates with multiple serotypes, suggesting that capsular switching events could have occurred (Table 2, Fig. 1). PFGE type 12 was the most prevalent (28%) and included isolates belonging to serotype 3 and corresponding to the clone Netherlands$^{3}$-31/ST180. The second most frequent (15%) was PFGE type 37 comprising penicillin-susceptible, multiresistant isolates of four different serotypes, belonging to the clone Greece$^{2}$-30/ST193 (Table 2, Fig. 1).

Among 39 CF isolates, eight and 12 different restriction profiles were identified for *php2b* and *php2x*, respectively (Table 2). Only the WT *php2b* restriction profiles 2b-1 and 2b-2 were found among the 32 penicillin-susceptible isolates (Table 2). The majority of PNSSP isolates within each PFGE type displayed unique *php2b* and *php2x* profiles (Table 2).

All isolates yielded a positive PCR for *pspA*, and 20 different restriction profiles were obtained (Table 2). Overall, 13 different *pspA* sequences, encompassing approximately 300–350 bp, were identified, and were 100%
Fig. 1. Phylogenetic analysis of PFGE profiles obtained from the 44 pneumococcal CF isolates. The dendrogram was reconstructed with PFGE profiles by similarity and clustering analysis using UPGMA and the Dice coefficient. The scale above the dendrogram indicates percentage genetic similarity. The ID strain code, with the serotype in parentheses, and the clone defined as PFGE type/PMEN reference clone/ST are indicated on the right. Duplicate strains obtained from the same patient with identical characteristics are indicated in italics.
### Table 2. Phenotypic and genotypic characteristics of pneumococcal clones recovered from CF patients

<table>
<thead>
<tr>
<th>PFGE type (no. isolates)</th>
<th>Corresponding PMEN clone and/or CC</th>
<th>Serotype (no. isolates)</th>
<th>Antibiotic non-susceptibility profile (no. isolates)</th>
<th>( pbp2b ) RFLP type (no. isolates)</th>
<th>( pbp2x ) RFLP type (no. isolates)</th>
<th>( pspA ) RFLP type (no. isolates)</th>
<th>PspA clade (no. isolates)/family*</th>
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<td>12 (11)</td>
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<td>4 (2)</td>
<td>3 (2)/2 (2)</td>
</tr>
<tr>
<td>59 (2)</td>
<td>Colombia(^{23F}/)ST338</td>
<td>23A (1), 23B (1)</td>
<td>PEN, CTX, ERY, CLI, TET (1); PEN (1)</td>
<td>19 (1), 38 (1)</td>
<td>11 (1), 29 (1)</td>
<td>17 (1), 18 (1)</td>
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<tr>
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<td>Sweden(^{15A-25}/)ST63</td>
<td>15A(1)</td>
<td>PEN (1)</td>
<td>10 (1)</td>
<td>46 (1)</td>
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<td>Spain(^{9C}/)ST156</td>
<td>14 (1)</td>
<td>PEN, CTX, ERY, CLI, TET (1)</td>
<td>6 (1)</td>
<td>2 (1)</td>
<td>5 (1)</td>
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<td>England(^{14-9}/)ST9</td>
<td>14 (1)</td>
<td>ERY (1)</td>
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<td>1 (1)</td>
<td>11 (1)</td>
<td>1 (1)/1 (1)</td>
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<td>CC460</td>
<td>35B (1)</td>
<td>PEN (1)</td>
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<td>CC1262</td>
<td>15B (2)</td>
<td>ERY, CLI, TET (1)</td>
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<td>20 (1)</td>
<td>PEN (1)</td>
<td>1 (1)</td>
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<td>ND</td>
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<td>PEN (1)</td>
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<td>3 (1)/2 (1)</td>
</tr>
<tr>
<td>95 (1)</td>
<td>6C (1)</td>
<td>2 (1)</td>
<td>ERY, CLI, TET (1)</td>
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<td>1 (1)</td>
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<tr>
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<td>ND</td>
<td>6A (1)</td>
<td>ERY, CLI, TET (1)</td>
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<td>1 (1)/1 (1)</td>
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<tr>
<td>97 (1)</td>
<td>ND</td>
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<td>ERY, CLI, TET (1)</td>
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<td>1 (1)</td>
<td>16 (1)</td>
<td>3 (1)/2 (1)</td>
</tr>
</tbody>
</table>

ND, Not determined; RFLP, restriction fragment length polymorphism. For other abbreviations, see Table 1.

*\( pspA \) sequences, encoding PspA clades and encompassing approximately 300–350 bp, were 100% identical to sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
identical to sequences available in GenBank. PspA clades 1 and 2 belonging to family 1 predominated (24 out of 39, 61.5%). Only two isolates carried PspA clade 3, whilst none showed PspA family 3. No significant associations were found between serotypes and PspA types. However, the majority of isolates within each clonal group shared identical PspA types (data not shown).

**Biofilm formation**

A large variability in biofilm formation was observed among strains (biofilm index range, 0.501–7.557; coefficient of variation, 60.3%) (Fig. 2). No correlation was observed between biofilm level and serotype, clone or antibiotic resistance (Fig. 3).

**DISCUSSION**

The pathogenic role of *S. pneumoniae* in CF patients is poorly understood (Cantón & del Campo, 2010; LiPuma, 2010), and the few available data point to a secondary role of *S. pneumoniae* in CF lung infection and deterioration (Gilligan, 1991). Therefore, we characterized pneumococcal isolates from CF children, a group of patients where the selective pressure exerted by antibiotics and by pneumococcal vaccination represents an important determinant for the epidemiology of this bacterial species.

A retrospective analysis of the patients’ medical charts did not disclose clinical evidence of pneumonia or exacerbation in any of the patients; therefore, a pathogenic role could not be ascribed to the pneumococcal isolates and their recovery was suggestive of colonization episodes. Laboratory data revealed that *S. pneumoniae* was associated with other common bacterial pathogens in the majority of the patients. Unlike *P. aeruginosa* and other typical CF pathogens, *S. pneumoniae* is not known as a persistent colonizer and is considered a transient organism in CF patients ( Renders *et al.* , 2001). However, we found that nine patients had multiple isolations of pneumococci from respiratory samples collected at different times. Molecular genotyping revealed that four patients were colonized by the same strain, whilst the remaining five were colonized by different strains. From our data, it was not possible to establish whether the former four patients were persistently colonized with the same strain or had acquired a new strain of the same clone. This was especially true for the two patients persistently colonized with serotype 3, as this serotype is commonly associated with CF children.

In this study, the proportion of PNSSP was approximately 18%, corresponding to the proportion observed among invasive isolates in the PCV7 era in Italian children ( Gherardi *et al.* , 2012; A. Pantosti, unpublished data), with only one PNSSP isolate of serotype 14 being fully penicillin resistant. Conversely, more than half of the isolates were resistant to erythromycin, clindamycin and tetracycline. This correlates with the high use of macrolides in these patients and with the evidence that in *S. pneumoniae* resistance determinants to these three antibiotic classes generally co-exist in the same genetic element; hence, macrolide use can co-select different antibiotic classes ( Varaldo *et al.* , 2009). No isolate was...
resistant to fluoroquinolones, probably due to the uncommon use of fluoroquinolones in children and to the rare occurrence of fluoroquinolone resistance among pneumococci. The susceptibility pattern observed in CF isolates was similar overall to that observed in IPD isolates from children (unpublished data).

Little information is available on the capsular types and genotypes of pneumococcal strains from CF patients (del Campo et al., 2005; Maeda et al., 2011). In our population, serotype 3 was the most common pneumococcal serotype comprising 30 % of the isolates, followed by serotypes 6C, 23A and 15B. Our findings are consistent with studies indicating that, in Italy, serotype 3 is the most common serotype among pediatric isolates from bacteraemic pneumonia and nasopharyngeal carriage (Ansaldi et al., 2012; Esposito et al., 2012; Camilli et al., 2013). Although generally associated with IPD and a high case-fatality rate in older children and adults (Henriques et al., 2000; Rosen et al., 2011), in young children serotype 3 seems to be associated with carriage and low invasive potential (Beall et al., 2006), and this is also the case in CF children.

In our study, we observed an overall coverage of PCV7 and PCV13 of approximately 8 and 46 %, respectively, with the higher coverage of PCV13 mainly being due to inclusion of serotype 3. Nevertheless, some studies aimed at assessing the impact and effectiveness of PCV13 on pneumococcal invasive and carriage isolates in PCV13-vaccinated children found that this vaccine showed limited efficacy in the reduction in pneumococcal invasive and non-invasive diseases and carriage caused by serotype 3 (Dagan et al., 2013; Richter et al., 2013). This phenomenon could be due to the thick capsule of this serotype, which may render it poorly immunogenic. It might also be explained by the high prevalence of serotype 3 pneumococci among older children and adults, which represent groups that do not receive the vaccine. It will be necessary in the future to monitor in more detail whether the lower immune response elicited by serotype 3 is associated with an impact on serotype 3 disease. In this regard, a study should be also mentioned that evaluated the trend for IPD in children after the PCV13 introduction in USA, which conversely provided early evidence for the effectiveness of this
conjugate vaccine in preventing IPD caused by serotype 3 (Kaplan et al., 2013). In studies of pneumococcal naso-
pharyngeal isolates from healthy Italian children, the majority of isolates were PCV13-NVSs (Camilli et al.,
2013; Zuccotti et al., 2014). One of these studies, which comprised unvaccinated, PCV7-vaccinated and PCV13-
vaccinated children, reported a reduction in the additional PCV13-VS colonization, especially 19A, in PCV13-vacci-
nated children, with a shift in the pneumococcal serotype composition in pneumococcal carriage isolates from a
mix of PCV7-VSs, PCV13-VSs and PCV13-NVSs in non-vaccinated infants to almost all PCV13-NVSs in both
PCV7- and PCV13-vaccinated children (Zuccotti et al., 2014). The difference could be explained by the fact that
these studies included children vaccinated with PCV13, whilst CF patients included in this investigation were
vaccinated with PCV7. These studies revealed that 6C was the most prevalent serotype, followed by 19A, 23A and 24F
(Camilli et al., 2013; Zuccotti et al., 2014). We found that serotypes 6C and 23A were also well represented among CF
isolates. The high proportion of PCV13-NVSs could raise some concern regarding the future efficacy of PCV13
immunization in this population.

A typical feature of serotype 3 pneumococci is their mucoid appearance, a unique characteristic of this serotype. The
finding that the most common serotype among CF isolates has a mucoid phenotype could be explained by the better
survival of mucoid strains in the CF pulmonary environ-
ment, a common trait of other CF pathogens involved in
chronic lung infection (LiPuma, 2010). However, we cannot
rule out that the appearance of serotype 3 colonies provides
an advantage for their detection and identification, whilst
other types of pneumococcal colonies could be overlooked
among z-haemolytic streptococci, thus affecting our results.

Irrespective of mucoid appearance, all isolates tested pro-
duced biofilm on polystyrene, thus confirming that the
ability to produce biofilm is a constitutive property of pneu-
 mococci (Camilli et al., 2011). Biofilm formation efficiency differed significantly among the strains tested and was not correlated with serotype, suggesting that in S. pneumoniae a specific but variable adhesin – or even a
strain-specific array of several adhesins – rather than capsular polysaccharide structure might be responsible for
determining efficiency in biofilm formation.

In contrast, we have to consider that biofilm formation is
a multifactorial phenomenon involving a multiplicity of genes
whose expression is significantly affected by environmental
stimuli. In this regard, the serotypes we tested might differ for
biofilm amount and architecture under different experimental
settings, as observed previously by Allegrucci et al. (2006)
using a continuous-flow biofilm reactor system. Similarly to
Vandevelde et al. (2014), no significant correlation was also
seen between biofilm amount and susceptibility to antibiotics
among the isolates investigated. Further studies focusing on
these topics are needed to understand better the role of
biofilm formation in pneumococcal pathogenicity.

By PFGE analysis, we found that approximately 80 % of the
CF strains belonged to a PMEN international clone, the
most prevalent being the multiresistant, penicillin-suscept-
bile clone PFGE 12/Netherlands3-31/ST180, followed by
clone PFGE 37/Greece21,30/ST193. Clone Netherlands3-31/
ST180 is known to be predominant among both invasive
and carriage serotype 3 strains from several countries. In
the USA, this clone was one of the most predominant
PCV7-NVS clones that expanded after the introduction of
PCV7 (Beall et al., 2006). In Italy, Netherlands3-31/ST180
was present among invasive antibiotic-resistant isolates
before and after PCV7 introduction (Dicuonzo et al., 2002;
Gherardi et al., 2009, 2012). This clone was shown to be
associated with higher carriage rates and low invasive
potential in young children (Brueggemann et al., 2004), as
also appears to be the case in CF patients.

PBPs 2x and 2b are primary targets for β-lactams and their
mutations confer low-level resistance (Grebe & Hakenbeck,
1996). PBP 2b mutations can be selected by penicillins only,
whilst PBP 2x plays a role in resistance to cephalosporins
(Grebe & Hakenbeck, 1996; Muñoz et al., 1992). PBP 1a is
involved in high-level resistance to cephalosporins (Muñoz
et al., 1992). In this study, we confirmed the usefulness of
pbp gene restriction profiling as a predictor of penicillin
susceptibility, as distinct restriction profiles were found in
penicillin-susceptible and penicillin-resistant isolates, and as
a typing marker for PNSSP.

By pspA restriction profiling and sequencing, we found both
PspA family 1 and 2 among CF isolates with a
predominance of PspA family 1, clades 1 and 2. Consistent
with previous observations (Rolo et al., 2009), our findings
suggest that PspA family 1 and 2 antigens are promising
candidates for consideration in future PspA-based vaccine
formulations.

To the best of our knowledge, this is the first Italian study
describing the characteristics of S. pneumoniae isolates
from CF patients. Its main limitations are the small sample
size and the inclusion of isolates from a single medical
centre, which could affect the results obtained, as possible
interactions between CF patients attending the same
centre cannot be excluded. Further studies are needed
involving a larger number of strains obtained from dif-
ferent CF centres in Italy in order to establish the clinical
significance of pneumococci in CF patients and the role of
pneumococcal serotypes and clones in exacerbation of this
disease, and to assess the impact of the new conjugate
vaccine, PCV13, in preventing pneumococcal colonization
and disease in these patients.

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