Increase of pneumococcal serotype 19A in Italy is due to expansion of the piliated clone ST416/CC199

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The emergence of Streptococcus pneumoniae serotype 19A, following use of the heptavalent pneumococcal conjugate vaccine (PCV7), has been favoured by multiple antibiotic resistance of this serotype and by other unknown factors. The aim of this study was to examine 19A isolates from invasive pneumococcal diseases (IPD) obtained before and after PCV7 implementation to ascertain which characteristics, including the presence of pili, might have favoured the emergence of this serotype in Italy. All S. pneumoniae isolates from IPD collected at the Italian National Institute of Health in the years 2001–2003 and 2006–2009 were serotyped. The 19A isolates were submitted to antimicrobial susceptibility testing by Etest and were genotyped by a combination of pulsed field gel electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST). The presence of the pilus islets PI-1 and PI-2 was detected by PCR assays targeting a marker gene in each islet. The proportion of 19A isolates from IPD significantly increased from 4 % in 2001–2003 to 12 % in 2006–2009. This was largely due to the expansion of a clone characterized by sequence type (ST) 416, clonal complex (CC) 199, already present in Italy before PCV7 implementation. This clone included isolates susceptible to penicillin and containing PI-1 genes. Other CCs contributed to the emergence of serotype 19A: CC63 and CC193, already present in 2001–2003, and new-emerging CCs or clones such as CC230, CC320 and ST5204, that include drug-resistant and/or pilus-positive isolates. The expansion of serotype 19A in Italy might have been favoured not only by antibiotic resistance, but also by other bacterial factors such as the presence of pili.

INTRODUCTION

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the United States in 2000 caused a reduction in the number of cases of invasive pneumococcal disease (IPD) not only in the vaccinated paediatric population but also in unvaccinated children, adults, and elderly persons, due to the indirect protective effects of the vaccine, the so-called ‘herd immunity’ (Whitney et al., 2003). In the same time period, an increase in IPD cases caused by serotypes not included in PCV7 (non-vaccine serotypes, NVS) was reported (Hsu et al., 2009). Most of the increase in NVS was due to a single serotype, serotype 19A, which before PCV7 vaccination was a low-frequency serotype (Hausdorff et al., 2000). In the US, the rate of IPD caused by serotype 19A in children under 5 years of age increased from 2.6 cases per 100 000 in 1998–1999 to 11 cases per 100 000 in 2007, making 19A the serotype most commonly isolated in that age group (Pilishvili et al., 2010).

Emergence of serotype 19A was observed, to variable extents, in all the countries where pneumococcal vaccination programs were implemented. In France, the percentage of 19A isolates recovered from meningitis and bacteraemia in children under the age of 2 years more than doubled between the periods before and after vaccine implementation (from 8 % to 19 % in meningitis cases and

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Abbreviations: PCV7, heptavalent pneumococcal conjugate vaccine; IPD, invasive pneumococcal diseases.
from 11% to 27% in bacteraemia cases) (Lepoutre et al., 2008). In Germany, the proportion of 19A isolates from IPD in children under the age of 16 increased from 2.8% in the pre-vaccination period to 15.0% in the years 2010–2011 (van der Linden et al., 2013). In Spain and Portugal, even relatively modest vaccination coverage rates significantly affected the serotypes found in IPD of children and adults (Aguiar et al., 2008a; Muñoz-Almagro et al., 2008).

Antibiotic use likely provides a survival advantage to antibiotic-resistant 19A clones, as demonstrated in South Korea, where the spread of the multidrug-resistant CC320 in the absence of a vaccination program was attributed to antibiotic pressure (Choi et al., 2008). Similarly, in Spain, an increasing trend in IPD caused by serotype 19A in the decade antecedent PCV7 introduction was ascribed to the antibiotic resistance of this serotype (Fenoll et al., 2009).

Besides antibiotic resistance, other bacterial factors might contribute to the success of a particular serotype. Pili are bacterial fibrillar appendages recently discovered in streptococcal species, including S. pneumoniae (Kreikemeyer et al., 2011; Barocchi et al., 2006). Two structurally and antigenically distinct pilus types, pilus-1 and pilus-2, have been identified in pneumococcus. Pilus-1 has been demonstrated to be involved in the initial stages of colonization and infection in animal models (Barocchi et al., 2006; Nelson et al. 2007). The genes encoding pilus-1 and pilus-2 are contained in a 14 kb pathogenicity island, the pilus islet (PI) 1, while the genes encoding pilus-2 are contained in a different chromosomal region (PI-2) of approximately 6.5 kb. In a global collection of S. pneumoniae, PI-1 and PI-2 are present in approximately 30% (Moschioni et al., 2008) and 16% (Bagnoli et al., 2008) of the isolates, respectively. Since pilus-1 proteins have been demonstrated to be protective against pneumococcal infections (Gianfaldoni et al., 2007), they are regarded as potential components for a pneumococcal protein vaccine.

PCV7 was introduced in Italy in 2001 and was gradually incorporated into the regional vaccination programmes. Vaccination coverage in children 24 months of age increased on a national basis from less than 3% in 2003 to 55% in 2008 (ICONA Working Group, 2009).

In this study, we examined S. pneumoniae isolates from IPD, obtained before and after the implementation of PCV7, to ascertain the change in the frequency of serotype 19A isolates. In order to understand the factors driving the increase in serotype 19A, the antibiotic susceptibility patterns, the clonal structure, and the presence of pil encoding genes were determined.

**METHODS**

S. pneumoniae isolates were obtained from IPD (meningitis, bacteraemic pneumonia, and bacteraemia with or without focus) in the framework of the Italian nationwide surveillance of invasive bacterial diseases (http://www.simii.iss.it/files/Report_MBLI.pdf). All the isolates from blood or cerebrospinal fluid originating from hospital laboratories all over the country and collected at the Italian National Institute of Health in the years 2001–2003 (pre-PCV7 implementation) and 2006–2009 (post-PCV7 implementation) were examined. Duplicate isolates from the same patient were excluded.

Identification of S. pneumoniae was confirmed by colony morphology and the optochin test. In case of ambiguous results, a bile solubility test was also performed.

The isolates were serotyped by latex agglutination and the Quellung reaction using the antisera produced by the Statens Serum Institute of Copenhagen, Denmark. Susceptibility to penicillin (PEN), ceftriaxone (CRO), erythromycin (ERY), clindamycin (CLI), tetracycline (TET) and chloramphenicol (CHL) was assayed by Etest (AB Biodisk). The results were interpreted following the breakpoints recommended by EUCAST for meningitis (European Committee on Antimicrobial Susceptibility Testing, 2013) considering isolates showing PEN MIC of >0.06 µg ml⁻¹ as PEN-nonsusceptible S. pneumoniae (PNSSP). For epidemiological purposes only, the definitions of low-level PEN resistance (MIC, 0.12 to 1 µg ml⁻¹) and high-level PEN resistance (MIC, >1 µg ml⁻¹) were used. Genes responsible for resistance to ERY and TET were detected by PCR as previously described (Monaco et al., 2005; Del Grosso et al., 2007).

The presence of PI-1 and PI-2 was detected by PCR assays targeting a marker gene in each islet. The thr gene was targeted for PI-1 using primers RLRA-up and RLRA-dn (Aguiar et al., 2008b); the pitB gene was targeted for PI-2 using primers P08-for and P08-rev (Bagnoli et al., 2008). PI-1 presence/absence was also confirmed using primers designed on conserved regions on the boundaries of PI-1 (459 for, 470 rev), as previously described (Moschioni et al., 2008).

Isolates were genotyped by a combination of pulsed field gel electrophoresis (PFGE) and Multi Locus Sequence Typing (MLST) (Gherardi et al., 2012). Briefly, PFGE was performed in all isolates following digestion of total bacterial DNA by Smal. Isolates showing PFGE profiles that differed by more than six bands were assigned to a different PFGE type. MLST was performed according to the method recommended at the MLST website (http://spneumoniae.mlst.net) on representative isolates (at least one isolate out of three, including one isolate for each PFGE type and for each antibiotic susceptibility profile). In order to identify pneumococcal clones, STs were compared to those of the Pneumococcal Molecular Epidemiology Network (PMEN) (http://www.sph.emory.edu/PMEN/) and assigned to Clonal complexes (CCs) using the eBURST software at the MLST website (http://spneumoniae.mlst.net).

**RESULTS**

In the years 2001–2003, 428 isolates were obtained from IPD cases. Of these, 227 (53.0%) belonged to serotypes contained in PCV7 (vaccine serotypes, VS) and 201 (47.0%) to NVS (Table 1). Among isolates obtained in 2006–2009, VS decreased to 28.2% (203/721) and NVS increased to 71.8% (518/721). The increase in NVS was statistically significant and was associated with statistically significant increases of serotypes 1, 19A and 24F (Table 1). Serotype 19A was the most abundant NVS in 2006–2009. Only 1 of 17 serotype 19A isolates (5.8%) from 2001–2003 was PNSSP, showing a low-level PEN resistance, while 17 of 87 serotype 19A isolates (19.5%) from 2006–2009 were PNSSP, with three isolates showing high-level PEN resistance (Table 2). Resistance to ERY was present in seven (41.1%) of the isolates from 2001–2003 and in 56
(64.3%) of the isolates from 2006–2009 and was commonly due to the presence of the \textit{erm}(B) gene, conferring also CLI resistance (MLSB phenotype) (Table 2). Resistance to TET, frequently associated with the MLSB phenotype, increased from 41.1% in 2001–2003 to 59.7% in 2006–2009 and was due to the presence of \textit{tet}(M). All the isolates from both periods were susceptible to CHL.

By PFGE typing, six and 14 different PFGE types were found among isolates from 2001–2003 and 2006–2009, respectively, with three PFGE types recovered from both periods. On the basis of PFGE results, 43 representative isolates were chosen as described in the Methods, and studied by MLST. Overall there was an excellent correspondence between PFGE and MLST results as previously observed (Gherardi et al. 2007, 2012). eBURST analysis identified three CCs among the 17 isolates from 2001–2003, and seven CCs and one singleton group among the 87 isolates from 2006–2009 (Table 2).

In the isolates from 2001–2003, the most abundant clone, including 13 of 17 isolates (76.4%), was characterized by ST416, a subgroup founder inside CC199, related to the PMEN clone Netherlands15B-37 (Gherardi et al. 2007). Two other minor CCs were identified: CC63, related to Sweden15A-25, and CC193, related to Greece21-30.

Also among isolates from 2006–2009, CC199 was the major CC, comprising 54 isolates out of 87 (62.0%) including strains belonging to ST416, or to its single locus variants (SLV) (ST2343, ST3008, ST7118) or double-locus variant (DLV) (ST686), as shown in Table 2. The second and third most common CCs were CC36 and CC193, already present among isolates from 2001–2003. The other CCs found were CC230, related to Denmark14-32, CC320, related to Taiwan19F-14, and ST5204, indicated by eBURST as a singleton, that is an individual, unlinked ST (Table 2).

The antibiotic resistance pattern appeared rather consistent for each CC. ST416/CC199 was represented by PEN-susceptible isolates, but over 50% of the isolates from 2006–2009 were resistant to ERY, CLI, and TET (Table 2). CC63 was represented by low-level PEN resistance while isolates belonging to CC193 were uniformly PEN susceptible; in both CCs, isolates were mostly resistant to ERY, CLI, and TET. As for the newly emerged CCs, isolates belonging to CC230 showed low-level PEN resistance, and resistance to ERY, CLI, and TET (Table 2). CC320 showed a typical multidrug-resistant phenotype including high-level PEN resistance, MLSB phenotype, and TET resistance due to the presence of double macrolide resistance genes, \textit{erm}(B) and \textit{mef}(E), along with \textit{tet}(M). All isolates belonging to ST5204, except one, were susceptible to the antibiotics tested (Table 2).

The presence of the pilus islets was strictly clone-related: all the isolates belonging to ST416 and its closely related SLV and DLV carried PI-1 while two other groups of isolates, CC320 and the ST5204 group, carried PI-1 in association with PI-2. Isolates belonging to other CCs did not contain PI genes. Overall, 72.4% of the 19A isolates from 2006–2009 were PI-1 positive (Table 2).

**DISCUSSION**

Similar to the occurrence in other countries where pneumococcal vaccination was adopted (Whitney et al., 2003; Miller et al., 2011), the percentage of NVS among strains causing IPD in Italy greatly increased after PCV7 implementation, reaching over 70% of the isolates recovered in the years 2006–2009. Serotype 19A was the third most common non-PCV7 serotype in the pre-vaccine era, but in the post-vaccine period became the principal

<table>
<thead>
<tr>
<th>Serotype</th>
<th>2001–2003 (no.=428)</th>
<th>2006–2009 (no.=721)</th>
<th>( P)-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>19A</td>
<td>17 (4.0)</td>
<td>87 (12.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>1</td>
<td>16 (3.7)</td>
<td>77 (10.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>3</td>
<td>38 (8.9)</td>
<td>60 (8.3)</td>
<td></td>
</tr>
<tr>
<td>7F</td>
<td>24 (5.6)</td>
<td>56 (7.8)</td>
<td></td>
</tr>
<tr>
<td>22F</td>
<td>11 (2.6)</td>
<td>25 (3.5)</td>
<td></td>
</tr>
<tr>
<td>24F</td>
<td>1 (0.2)</td>
<td>22 (3.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6A</td>
<td>9 (2.1)</td>
<td>19 (2.6)</td>
<td></td>
</tr>
<tr>
<td>15B</td>
<td>11 (2.6)</td>
<td>15 (2.1)</td>
<td></td>
</tr>
<tr>
<td>10A</td>
<td>8 (1.9)</td>
<td>13 (1.8)</td>
<td></td>
</tr>
<tr>
<td>11A</td>
<td>7 (1.6)</td>
<td>13 (1.8)</td>
<td></td>
</tr>
<tr>
<td>12F</td>
<td>7 (1.6)</td>
<td>10 (1.4)</td>
<td></td>
</tr>
<tr>
<td>15A</td>
<td>1 (0.2)</td>
<td>10 (1.4)</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td>51 (11.9)</td>
<td>111 (15.4)</td>
<td></td>
</tr>
<tr>
<td>All NVS</td>
<td>201 (47.0)</td>
<td>518 (71.8)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of the 19A isolates obtained before (2001–2003) and after (2006–2009) the implementation of PCV7

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>ST</th>
<th>CC</th>
<th>PMEN clone</th>
<th>No. (%) of isolates resistant to*</th>
<th>ERY-R† genes (no. of isolates)</th>
<th>No. (%) of isolates harbouring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PEN</td>
<td>CRO</td>
<td>ERY</td>
</tr>
<tr>
<td>2001–2003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>416</td>
<td>199</td>
<td>Netherlands15B-37/ST199</td>
<td>0</td>
<td>0</td>
<td>3 (23.0)</td>
</tr>
<tr>
<td>2</td>
<td>63, ST_new†</td>
<td>63</td>
<td>Sweden15A-25/ST63</td>
<td>1 (50)§</td>
<td>0</td>
<td>2 (100)</td>
</tr>
<tr>
<td>2</td>
<td>193</td>
<td>193</td>
<td>Greece13-30/ST193</td>
<td>0</td>
<td>0</td>
<td>2 (100)</td>
</tr>
<tr>
<td>2006–2009</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>54</td>
<td>416, 686, 2343, 3008, 7118</td>
<td>199</td>
<td>Netherlands15B-37/ST199</td>
<td>0</td>
<td>0</td>
<td>33 (61.1)</td>
</tr>
<tr>
<td>9</td>
<td>63, 3816</td>
<td>63</td>
<td>Sweden15B-25/ST63</td>
<td>8 (88.8)§</td>
<td>0</td>
<td>8 (88.8)</td>
</tr>
<tr>
<td>8</td>
<td>193, 7120</td>
<td>193</td>
<td>Greece13-30/ST193</td>
<td>0</td>
<td>0</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>6</td>
<td>5204</td>
<td>-I</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>1 (16.6)</td>
</tr>
<tr>
<td>5</td>
<td>276, 2013</td>
<td>230</td>
<td>Denmark14-32/ST230</td>
<td>5 (100)§</td>
<td>2 (40.0)</td>
<td>4 (80.0)</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
<td>320</td>
<td>Taiwan19F,41/ST236</td>
<td>3 (100)§</td>
<td>3 (100)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>1</td>
<td>5460</td>
<td>15</td>
<td>England14-9/ST9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1131</td>
<td>156</td>
<td>Colombia23F-26/ST338</td>
<td>1 (100)§</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*PEN: penicillin; CRO: ceftriaxone; ERY: erythromycin, CLI: clindamycin, TET: tetracycline.
†ERY-R: erythromycin resistance.
§ST not assigned due to a deletion of xpt (Gherardi et al., 2007).
||Singleton.
¶High-level penicillin resistance.

Increase of pneumococcal serotype 19A in Italy
Serotype 19A is a genetically heterogeneous serotype. It is found associated with five major CCs and with a number of other CCs related to different predominant serotypes (Reinert et al., 2010), indicating that capsular switching has extensively occurred between serotype 19A and these other serotypes. Capsular switching at the DNA sequence level was documented in isolates from the United States, showing exchange of capsular genes from serotype 19A to serotype 4 isolates belonging to ST695 (Brueggemann et al., 2007). Similar vaccine escape strains were identified in Italy (Ansaldi et al., 2011).

The increase of serotype 19A in Italy was mainly associated with a clonal subgroup (ST416) of CC199, already established in our country since at least 2001 (Gherardi et al., 2007), and which expanded in number and proportion.

Since ST416 isolates were PEN susceptible and only 60% of the isolates were resistant to macrolides, we wondered which other characteristics beyond antibiotic resistance might have driven the emergence of this serotype. The detection of PI-1 in isolates belonging to ST416 and to closely related STs was unexpected, since CC199 isolates, in particular the group founder ST199, have been reported to be PI-1 negative (Basset et al., 2007; Regev-Yochay et al., 2010; Moschioni et al., 2008; Selva et al., 2012). Our finding confirmed that the presence of the PI-1 was strictly clonal, that is associated with the ST and not necessarily with the CC, in accordance with previous observations (Moschioni et al., 2008; Aguiar et al., 2008b). In addition, the presence of PI-1 in the PEN-susceptible ST416 isolates was at odds with the association between the presence of pilus islets and antibiotic resistance, reported by other studies (Moschioni et al., 2008; Selva et al., 2012). Although we cannot exclude that ST416/CC199 clone expansion and presence of pili could be a coincidence, pili can represent a competitive advantage for this clone, favouring colonization of the human nasopharynx (Barocchi et al., 2006). In this way, ST416/CC199 isolates can occupy the niche left by PCV7 serotypes, outnumbering other NVS, and eventually causing infections in susceptible hosts.

PI-2 was present in two minor 19A groups associated with PI-1: in the worldwide spread CC320, as already reported (Bagnoli et al., 2008), and in the novel clone ST5204. The global success of the multidrug-resistant CC320, related to Taiwan19F-14, might depend on the combination of different factors, including antibiotic resistance (Pillai et al., 2009) and presence of both types of pili.

The same 19A clones recovered in Italy were also responsible for the increase in 19A serotypes in other countries, although their relative contribution varies greatly. In the US, CC199 was the most abundant clone among serotype 19A, comprising mainly isolates belonging to the group founder ST199 (Beall et al., 2011), that do not contain PI-1 (Moschioni et al., 2008). In the last years, the multidrug-resistant and piliated CC320 has greatly increased within serotype 19A isolates, ranking second close to CC199 (Beall et al., 2011).

In Germany, the most common 19A clone is CC199, and isolates of ST199 and ST416 are equally represented (van der Linden et al., 2013). In France, Spain and Portugal, the most abundant serotype 19A clone is ST276/CC230, related to Denmark14-32 (Mahjoub-Messai et al., 2009; Marimon et al., 2012; Aguiar et al., 2010), which is PEN-nonsusceptible and does not carry PIs (Moschioni et al., 2008). CC199 is the second largest CC in Spain, with isolates belonging mainly to the non-piliated founder ST199 (Marimon et al., 2012), while in Portugal and France, it represents only a small proportion of the 19A population (Mahjoub-Messai et al., 2009; Aguiar et al., 2010).

The success of serotype 19A is probably linked to its genetic diversity and to different factors in different countries. The interplay between antibiotic resistance and antibiotic use could play a role in certain geographical areas, but other bacterial characteristics, such as the presence of pili, can favour the spread and the establishment of a particular clone.

In the light of the implementation of the pneumococcal 13-valent vaccine, which includes serotype 19A, a decrease in the circulation of this serotype can be expected. However, further serotype replacement will probably occur, and studies to understand which factor(s) are the drivers of the emergence of new serotypes and clones remain important.

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