Prevalence of pilus genes in pneumococci isolated from healthy preschool children in Iceland: association with vaccine serotypes and antibiotic resistance

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Objectives: The objective of this study was to investigate the prevalence of pilus islets (pilus islet 1 (PI-1) and pilus islet 2 (PI-2)) in pneumococcal isolates from healthy Icelandic preschool children attending day care centres, prior to the introduction of conjugated pneumococcal vaccine, and the association of the pilus islets with vaccine serotypes and antibiotic resistance.

Methods: Nasopharyngeal swabs were collected from 516 healthy children attending day care centres in Reykjavik in March and April 2009. Infant vaccination was started in 2011, thus the great majority of the children were unvaccinated. Pneumococci were cultured selectively, tested for antimicrobial susceptibility and serotyped. The presence of PI-1 and PI-2 was detected using PCR.

Results: A total of 398 viable isolates were obtained of which 134 (33.7%) showed the presence of PI-1. PI-1-positive isolates were most often seen in serotype 19F (30/31 (96.8%) and were of clade I, and in 6B (48/58 (82.8%)) of clade II. PI-2-positive isolates were most common in serotype 19F (27/31 (87.1%); all of them were also PI-1 positive. Of the PI-1-positive and PI-2-positive isolates, 118 (88.1%) and 31 (81.6%), respectively, were of vaccine serotypes. Both PI-1 and PI-2 were more often present in penicillin-non-susceptible pneumococci (PNSP) than in penicillin-susceptible pneumococci (PI-1 in 41/58 (70.7%) and 93/340 (27.4%), respectively, and PI-2 in 28/58 (48.3%) and 10/340 (2.9%), respectively).

Conclusions: Genes for PI-1 and/or PI-2 in pneumococci isolated from healthy Icelandic children are mainly found in isolates of vaccine serotypes and in PNSP isolates belonging to multiresistant international clones that have been endemic in the country.

Keywords: pili, PI-1, PI-2, PNSP

Introduction

Streptococcus pneumoniae is a frequent colonizer of the human upper respiratory tract but also a major human pathogen that causes diverse infections ranging from acute otitis media to life-threatening invasive disease. The current conjugate vaccines are limited to few serotypes (10-valent: 1,4,5,6B,7F,9V,18C,19F,23F; and 13-valent: also 3,6A,19A). Capsular switching1 and increasing replacement of non-vaccine serotypes2 makes development of a serotype-independent vaccine made from pneumococcal proteins more important. A number of proteins have been considered in this context, among them the recently found pili.3,4

Pneumococcal pili affect their ability to adhere to human respiratory epithelial cells.5–8 Furthermore, murine models suggest that pili promote invasiveness of pneumococci by enhancing phagocytosis and prolonging the survival of pneumococci after phagocytosis.9

The two major types of pili are encoded by two genetic islets, pilus islet 1 (PI-1)4 and pilus islet 2 (PI-2).5 PI-1 is 14 kb and consists of seven genes: a transcription regulator gene rlrA; three genes coding for pili subunits, rrgA, rrgB and rrgC; and three genes coding for sortases, srtB, srtC and srtD.10 Its genetic variability can be determined from variable regions within the rrgB gene and is presented by three clade types.11 Reported prevalence of PI-1 in invasive disease, carriers, otitis media and global collections ranges from 10% to 35%.6,12–20

PI-2 is just over 6.5 kb and includes five genes: pitA coding for pilus subunits; pitB coding for the backbone protein; srtG1 and srtG2
Coding for sortases, and sipA coding for signal-peptidase-related product.6 Reported prevalence of PI-2 ranges from 0% to 21% in invasive disease, carriers, otitis media and global collections.5,12,15–17

Knowledge about the prevalence of piliated pneumococci in the nasopharynx of healthy children and in disease enhances the understanding of pneumococcal pathogenicity and is essential for evaluation of the feasibility of pil as a candidate for future vaccines. The aim of this study was to investigate the prevalence of PI-1 and PI-2 in pneumococcal isolates from healthy Icelandic preschool children attending day care centres, prior to implementation of conjugated pneumococcal vaccine, and to investigate the association of the pilus islets with vaccine serotypes and antibiotic resistance.

Methods

Bacterial isolates and serotyping

Nasopharyngeal swabs were collected from 516 healthy children attending 15 representative day care centres in the metropolitan area of Reykjavik. The children were considered healthy if they attended on the day of sampling. The samples were collected in March and April 2009 after obtaining informed consent from a parent/guardian contacted through the day care centres. Pneumococci were isolated from the swabs using conventional methods and their identification confirmed with PCR using primers for lytA, the association of the pilus islets with vaccine serotypes and antibiotic resistance.

Table 1. Nucleotide sequences of the primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5‘→3’)</th>
<th>Purpose</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>srtD for</td>
<td>CATGCTTTTTTCGCCCATTT</td>
<td>presence of PI-1</td>
<td>this study</td>
</tr>
<tr>
<td>srtD rev</td>
<td>CGTAGTAAACGCTGGCTTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFL for</td>
<td>CTCATTGACTACACAGATATCACCCTC</td>
<td>confirm absence of PI-1</td>
<td>modified from Aguiar et al. 33</td>
</tr>
<tr>
<td>PFL rev</td>
<td>AGCATATGCTACAACTGAAAAATATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P06 for</td>
<td>CGTGGATCAGGTGTTGACCTATGAAAA</td>
<td>presence of PI-2</td>
<td>Bagnoli et al. 6</td>
</tr>
<tr>
<td>P06 rev</td>
<td>GCCTCCTCTCTTAAATKACCTGTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1008 for</td>
<td>GCTGAGTCAGTTTGAACCGAGA</td>
<td>confirm absence of PI-2</td>
<td>Bagnoli et al. 6</td>
</tr>
<tr>
<td>1009 rev</td>
<td>TAAGGATCACAAAGTCGACGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clade I for</td>
<td>AACAGATGGGATGATTATAAATTG</td>
<td>clade I</td>
<td>Moschioni et al.11</td>
</tr>
<tr>
<td>Clade I rev</td>
<td>AATGGTAGACATTTCAATTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clade II for</td>
<td>AATCTATAAGTGCTGTCCTGA</td>
<td>clade II</td>
<td>Moschioni et al.11</td>
</tr>
<tr>
<td>Clade II rev</td>
<td>AATCCATCTACATATTCAAAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clade III for</td>
<td>GACAGATCAAGAGTCGGCCTTG</td>
<td>clade III</td>
<td>Moschioni et al.11</td>
</tr>
<tr>
<td>Clade III rev</td>
<td>CTGAGATCAAGGACCTGCTCACAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The presence or absence of PI-1 and PI-2 was determined using a modification of previously published PCR methods. In short, the presence of PI-1 was confirmed using the primers PFL for and PFL rev. The presence of PI-2 was detected using the primers P06 for and P06 rev. The absence of PI-1 was confirmed using the primers 1008 for and 1009 rev. All PI-1 were further classified to clades using specific primers for clades I–III.11 Primers used are listed in Table 1.

Statistical analysis

Pearson’s $\chi^2$ and Fisher’s exact test were used for statistical analysis and a $P$ value < 0.05 was considered significant.

Results

The children participating in the study were 1.2–6.3 years old; mean age = 4.1 years. Pneumococci were found in 372 of the samples (carriage rate, 72.1%). In some of the samples different colony appearances were noted. Each was sub-cultured and serotyped, revealing 32 samples with strains of two different serotypes. Thus, a total of 404 isolates were stored at $-80^\circ$C for the investigation. Of these, six were not available for pilus analyses, thus the final number of isolates tested for presence of pilus islet genes was 398. The most common serotype was 6B [58 (14.6%)], followed by 6A [48 (12.0%)], 23F [47 (11.8%)], 19A [43 (10.8%)] and 19F [31 (7.8%)]. There were 340 (85.4%) penicillin-susceptible pneumococci (PSP; MIC $\leq 0.1 \text{mg/L}$) and 58 (14.6%) penicillin-non-susceptible pneumococci (PNSP; MIC $>0.1 \text{mg/L}$). No isolates were fully resistant to penicillin (MIC $>2 \text{mg/L}$).

The prevalence of pneumococcal serotypes differed between the 15 day care centres, thus the majority of PNSP isolates of serotype 19F carrying PI-1 and PI-2 genes originated from children attending three of the day care centres, while they were very rarely detected in nine and not detected in one of the day care centres.

PI-1 and PI-2 detection

The presence or absence of PI-1 and PI-2 was determined using a modification of previously published PCR methods. In short, the presence of PI-1 was detected using the primers srtD for and srtD rev. The absence of PI-1 was confirmed using the primers PFL for and PFL rev. The presence of PI-2 was detected using the primers P06 for and P06 rev. The absence of PI-2 was confirmed using the primers 1008 for and 1009 rev. All PI-1 were further classified to clades using specific primers for clades I–III.11
not be confirmed by a positive PCR with the PFL primers. Distribution according to serotypes varied. Of the five most commonly carried serotypes, the proportional rates for PI-1 genes were highest in serotype 19F, where the vast majority, 96.8% (30/31), were PI-1 positive. However, the highest number of PI-1-positive isolates within a serotype was found in serotype 6B, but at lower rates, 82.8% (48/58). The PI-1 prevalence for serotype 9V was 90.0% (9/10), for serogroup 15 it was 68.4% (13/19) and for 6A it was 45.8% (22/48). Other serotypes carrying PI-1 genes were less commonly seen, including serotype 4, where 13/19 (68.4%) of the isolates were PI-1 positive. However, the highest number of PI-1-positive isolates belonging to the international clone Spain6B-2.

PI-2

The number of isolates that showed the presence of PI-2 genes was 38 (9.5%); 355 isolates (89.2%) were negative and no result was obtained for five isolates (1.3%), as the absence of PI-2 could not be confirmed by a positive PCR with the 1008 forward and 1009 reverse primers. Distribution according to serotypes varied. The highest number and prevalence of PI-2-positive isolates were seen in serotype 19F, where 87.1% (27/31) carried the genes. The presence of PI-2 was also detected in serogroup 11 where 41.2% (7/17) were positive, in serotype 6B 5.2% (3/58) and in 6A 2.1% (1/48). The presence of PI-2 was not detected in other serotypes. All of the isolates of serotype 19F that were PI-2 positive were also PI-1 positive. Isolates of serogroup 11 were only positive for PI-2, not PI-1, but one of the isolates of serotype 6B was positive for both islets (Figure 3).

**Association of the pilus islets with vaccine serotypes**

Of the 134 isolates positive for PI-1, 95 (70.9%) were of serotypes included in the 10-valent vaccine and a further 23 (88.1%) were of serotypes included in the 13-valent vaccine, thereof 22 of serotype 6A. Of the 16 PI-1-positive isolates that did not belong to vaccine types, 13 isolates were of serogroup 15. Of the 38 PI-2-positive isolates, 30 (78.9%) were of serotypes of the 10-valent vaccine, and additionally 1 (81.6%) of the 13-valent serotypes. There were 7 (18.4%) isolates of non-vaccine serotypes carrying PI-2 genes, all of serogroup 11. Isolates carrying pilus islet genes were more likely to belong to vaccine serotypes than not (P<0.001).

**Association of the pilus islets with antibiotic resistance**

PI-1 was more often present in PNSP, than in PSP, or in 70.7% (41/58) and 27.4% (93/340), respectively (P<0.001). The majority, or 28 isolates, of PI-1-positive PNSP were of serotype 19F, 5 were of serotype 6B, and 5 were of serotype 14. The majority, or 43 isolates, of PI-1-positive PSP were of serotype 6B, 21 were of serotype 6A, 13 were of serotype 15, and 9 were of serotype 9V. The same held true for carriage of PI-2 genes in PNSP and PSP. Thus, 48.3% (28/58) of the PNSP isolates were PI-2 positive, and 2.9% (10/340) of the PSP (P<0.001). Of the PI-2-positive PNSP, 26 isolates belonged to serotype 19F and 7 of the PSP belonged to serogroup 11 (Table 2). The isolates of serotype 19F carrying PI-1 genes of clade I and PI-2 genes were multiresistant, of several STs belonging to the clonal complex 236, and single-locus or double-locus variants of the international clone Taiwan19F-14. The PNSP of serotype 6B carrying PI-1 genes of clade II were of ST90, belonging to the international clone Spain6B-2.
Discussion

In this study we found genes of the islets encoding for pili of type 1 in one-third, and genes for pili of type 2 in every tenth isolate of pneumococci from healthy preschool children before pneumococcal vaccination was implemented in the infant vaccination programme.

The prevalence rate of 33.7% for PI-1 is in concordance with previously reported rates of 15%–35% in nasopharyngeal samples from healthy children.\textsuperscript{14,18,20} The rate is also comparable to reported rates of 20%–30% in acute otitis media\textsuperscript{12,15} and of 10%–33% in invasive disease.\textsuperscript{11,14,16,19} Furthermore, the prevalence rate of 9.4% for PI-2 is in concordance with previous studies on mixed, invasive and middle-ear collections reporting rates from 0% to 21%.\textsuperscript{6,12,15,16}

The vast majority of isolates carrying PI-1 genes belonged to serotypes included in the protein-conjugated vaccines. The majority of isolates of non-vaccine serotypes carrying PI-1 genes were of serogroup 15, but PI-1 genes were also detected in one isolate of serotype 6C and two non-typeable isolates. More than three-quarters of the PI-2-positive isolates belonged to the vaccine serotypes, the vast majority were of serotype 19F, but a few were of serotype 6B and one of serotype 6A. PI-2 presence in non-vaccine serotypes was only detected in serogroup 11. Thus, a vaccine including pili subunits would only provide additional protection to a limited number of serotypes, compared with the current protein-conjugated vaccines.

Vaccination has reduced rates of piliated pneumococci in invasive pneumococcal disease.\textsuperscript{18,29} However, re-emergence of both PI-1\textsuperscript{11} and PI-2\textsuperscript{29} is also reported to have occurred after a few years of vaccination due to piliated strains of non-vaccine types. Thus, we can expect that piliated pneumococci will become rare if the vaccination eradicates the vaccine types in healthy children. However, piliated strains of serogroups 11 and 15 could be of concern in Iceland in the future, as possible emerging clones of piliated non-vaccine serotypes in pneumococcal disease.

Since 2004 the most prevalent PNSP isolates in Iceland have been of serotype 19F, belonging to STs that are single-locus and double-locus variants of the international clone Taiwan\textsuperscript{19F-14} and belong to the clonal complex 236.\textsuperscript{30} The isolates of this clonal complex carry PI-1 genes of clade I and PI-2 genes, and are thus the main cause of the significantly higher rates of both PI-1 and PI-2 in PNSP isolates than in PSP. Before 2004 the most prevalent PNSP clone was of serotype 6B, ST90, belonging to the international clone Spain\textsuperscript{6B-2}.\textsuperscript{31} This clone is still present in Iceland, although at much lower rates, and carries PI-1 genes of clade II.

It is possible that piliated pneumococci select against other piliated pneumococcal strains.\textsuperscript{32} When a clone of a specific clade/class is prevalent for a period of time, it may also be possible that it induces herd immunity to that clade/class and thus selects against piliated pneumococci belonging to the same clade/class. If that is true, a successful new piliated clone would be likely to be of another clade/class. The fact that the piliated 19F clonal complex of clade class I replaced the piliated 6B clone of clade II as the most prominent PNSP clone in the country supports this theory.\textsuperscript{30} Possible pili vaccines would need to address that.

It might be considered a weakness in this study that we only tested for one of the genes, \textit{srtD}, as a marker for the presence of PI-1.\textsuperscript{2206} However, re-emergence of both PI-1\textsuperscript{11} and PI-2\textsuperscript{29} is also reported to have occurred after a few years of vaccination due to piliated strains of non-vaccine types. Thus, we can expect that piliated pneumococci will become rare if the vaccination eradicates the vaccine types in healthy children. However, piliated strains of serogroups 11 and 15 could be of concern in Iceland in the future, as possible emerging clones of piliated non-vaccine serotypes in pneumococcal disease.

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<table>
<thead>
<tr>
<th>Serotype</th>
<th>PNSP total</th>
<th>PNSP positive for pilus islet, n (%)</th>
<th>PSP total</th>
<th>PSP positive for pilus islet, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19F</td>
<td>28</td>
<td>28 (100) 26 (92.9)</td>
<td>3</td>
<td>2 (66.7) 1 (33.3)</td>
</tr>
<tr>
<td>6B</td>
<td>7</td>
<td>5 (71.4) 1 (14.3)</td>
<td>51</td>
<td>43 (84.3) 2 (3.9)</td>
</tr>
<tr>
<td>6A</td>
<td>1</td>
<td>1 (100) 1 (100)</td>
<td>47</td>
<td>21 (44.7) 0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>0 0 0</td>
<td>19</td>
<td>13 (68.4) 0</td>
</tr>
<tr>
<td>9V</td>
<td>0</td>
<td>0 0 0</td>
<td>10</td>
<td>9 (90.0) 0</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0 0 0</td>
<td>17</td>
<td>0 7 (14.2)</td>
</tr>
<tr>
<td>14</td>
<td>6</td>
<td>5 (83.3) 0 0</td>
<td>0</td>
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</tr>
<tr>
<td>NT</td>
<td>13</td>
<td>2 (15.4) 0 0</td>
<td>0</td>
<td>0 0</td>
</tr>
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<td>4</td>
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</tr>
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<td>1</td>
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<td>1 (2.4) 0</td>
</tr>
<tr>
<td>23F</td>
<td>1</td>
<td>0 0 0</td>
<td>46</td>
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</tr>
<tr>
<td>6C</td>
<td>0</td>
<td>0 0 0</td>
<td>3</td>
<td>1 (33.3) 0</td>
</tr>
<tr>
<td>Other</td>
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<td>100</td>
<td>0 0</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>41 28</td>
<td>340</td>
<td>93 10</td>
</tr>
</tbody>
</table>

NT, non-typeable.
We consider it a strength in this study that the absence of both pilus islets was confirmed with a positive PCR. The primers used flank the pilus islets and PCR can therefore only give a positive reaction when pilus islet genes are not present. However, in spite of repeated tests, we could not confirm the absence of PI-1 in three isolates and the absence of PI-2 in five isolates. In all instances, when no results were obtained for PI-1, the PCR worked well testing PI-2 and vice versa.

In conclusion, prior to pneumococcal vaccination, genes for PI-1 and/or PI-2 in pneumococci isolated from healthy Icelandic children were mainly found in isolates of vaccine serotypes and PNSP isolates belonging to multiresistant international clones that have been widely distributed in the country. A new prevailing clone replacing another in the same geographical area is likely to have pili of different clade class than the previous one and after implementation of vaccinations to be of a non-vaccine serotype.

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Transparency declarations
None to declare.

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