Epidemiology of Acute Otitis Media Caused by *Streptococcus pneumoniae* Before and After Licensure of the 7-Valent Pneumococcal Protein Conjugate Vaccine

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We studied, by pulsed-field gel electrophoresis, multilocus sequence typing and penicillin-binding protein 2b amplicon-restriction profiles, pneumococcal isolates recovered from children with acute otitis media during 1 January–31 December 1999 and 2001. The proportion of nonvaccine serogroups increased from 14.8% (13/88) to 36.5% (23/63) from 1999 to 2001 (/). Among children who received at least 2 doses of the pneumococcal 7-valent protein conjugate (PNC7) vaccine, 46.7% (7/15) of the isolates had nonvaccine serogroups, compared with 20.8% (26/125) of the isolates from children who did not receive the PNC7 vaccine (/). Overall, the serogroups involved in capsular switching were 6-19-NT, 6-14-35, 15-19, and the 19-Spanish 23F clone. In 1999 and 2001, 30.8% (4/13) and 26.1% (6/23) of the nonvaccine serogroups were implicated in capsular switching, respectively. Continued surveillance will be of great importance as the distribution of the PNC7 vaccine increases.

*Streptococcus pneumoniae* is the most common bacterial cause of acute otitis media (AOM) in children [1, 2], and 7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) cause the majority of infections [3]. Before the licensure of the pneumococcal 7-valent protein conjugate (PNC7) vaccine, 85% of pneumococcal middle-ear isolates from children with AOM were of vaccine serotypes or cross-reactive serotypes [3]. The PNC7 vaccine has been shown to prevent 6%–7% of episodes of AOM, regardless of cause [4, 5]. Among children who received 4 doses of the PNC7 vaccine, episodes of culture-confirmed pneumococcal AOM caused by either vaccine or cross-reactive serotypes were reduced by 51%, compared with children who did not receive the PNC7 vaccine. However, the number of episodes caused by nonvaccine serotypes increased by 33% among the children who were vaccinated, compared with children who did not receive the vaccine [4]. This may be due to an increase in genetically unrelated strains, an increase in capsular switching, or both. Either mechanism could result in an increase in AOM caused by *S. pneumoniae* despite vaccination.

*S. pneumoniae* organisms are able to switch their serotypes through serotype capsular transformation [6]. The capsular locus has been described for >15 serotypes and is composed of serotype-specific capsular genes, named alphabetically, which are flanked by the conserved *dexB* and *aliA* genes [7–21]. Capsular transformation has been shown to occur through large recombinational events within the capsular locus [22, 23]. Pneumococci that have switched capsular serotypes have been found, by various molecular subtyping techniques, including multilocus sequence typing (MLST) [22, 23] and pulsed-field gel electrophoresis (PFGE) [24, 25], to be highly genetically related. Serotype 19F...
and 19A variants of the Spanish 23F-1 clone, 1 of 15 international clones, have been described [24, 26]. In contrast, the penicillin-non-susceptible serotype 35B clone [27] has not yet been associated with serotype capsular transformation.

For the present article, we determined the serotype distribution of strains causing AOM and correlated these with both the calendar year and the number of doses of PNC7 vaccine that each child received. PFGE was done on all strains. MLST and restriction profiles of the penicillin-binding protein 2b (pbp2b) gene were done on the subset that appeared to have undergone serotype capsular transformation according to the results of PFGE.

**MATERIALS AND METHODS**

Middle-ear isolates were obtained from children with AOM who were treated at the Children’s Hospital of Pittsburgh between 1 January and 31 December 1999 and 1 January and 31 December 2001. These time periods were chosen to determine strains that were causing AOM before and after the licensure of PNC7 vaccine. A portion of the isolates were obtained from children enrolled in clinical studies of AOM by tympanocentesis or who had perforated their tympanic membranes acutely. The remaining isolates were recovered in the context of clinical care, either by tympanocentesis for failing to respond to antibiotics or myringotomy for tympanostomy tube insertion. The relative contribution of cases from the different sources was similar in 1999 and 2001. Receipt of the PNC7 vaccine in the preceding 30 days was defined as recent antibiotic use. Antibiotic use within 1 month before infection was defined as a vaccine dose. Antibiotic use within the preceding 30 days was defined as recent antibiotic use. Susceptibility testing and PFGE were done on all strains. Sequential isolates of *S. pneumoniae* were recovered from some children. Because tympanocentesis is done for clinical failures, persistent infections were defined as the recovery of 2 isolates of *S. pneumoniae* from the same child within 7 days. The remaining children with sequential isolates were classified as having relapses or reinfections. Strains were defined to be of the same PFGE type if the patterns had ≤3 bands of difference. Persistent or relapsing infections were defined as 2 strains with the same PFGE type and serotype. Reinfection was defined as 2 strains of different serotypes and/or PFGE types.

For strains that appeared to have undergone capsular switching by PFGE, MLST and restriction profiling of the *pbp2b* amplicon were done. The definition of capsular switching included serogroup, PFGE, and MLST. If ≥2 strains of different serogroups showed a ≤3-band difference in PFGE and/or 90% genetic relatedness on the dendrogram, the serogroup was repeated. If discrepant serogroup results were obtained, then the serogroup that was most consistent with the strain’s PFGE pattern was used in the analysis. If the serogroup was reproducible, MLST was done. If the strains shared at least 5 MLST alleles, the strains were classified as capsular switches. The *pbp2b* restriction profiles were done on all capsular switches, to further characterize the strains.

**Antibiotic susceptibility and serogrouping.** Susceptibility testing to penicillin, erythromycin, cefotaxime, chloramphenicol, clindamycin, and trimethoprim-sulfamethoxazole was done using the NCCLS [28] microbroth dilution method. Strains were tested for all serogroups using reagents from Statens Serum Institut.

**PFGE.** PFGE was done as described elsewhere [29, 30], with the following run parameters: 1–30 s for 18 h and 5–9 s for 8 h. After staining the gel with ethidium bromide, the image was captured on the Bio-Rad Gel Doc 2000 System (Bio-Rad). The genetic relatedness of strains was determined by analyzing the PFGE patterns using Molecular Analyst (Bio-Rad). Dendrograms were created using the unweighted pair-group method with arithmetic averages, the Dice coefficient, and a position tolerance of 1.5%.

**Polymerase chain reaction (PCR).** Strains were grown overnight on trypticase soy agar that contained 5% sheep blood. Genomic DNA was isolated using Prepman Ultra, according to the manufacturer’s instructions (Applied Biosystems). PCR primers for the restriction profiles were *pbp2b* F (GATCCCTCT-AAATGATTTCTCAGTGGCCTGT) and *pbp2b* R (GTCATTAG-GCTTAGCAAATAGGTTGGGAT) [24]. The 9700 Thermal Cycler (Perkin-Elmer) was used for a 30-μL reaction mixture that contained 1.5 mmol/L MgCl₂, 0.33 μmol/L each primer, 25 μmol/L each deoxyribonucleotide, 0.5 U of the thermostable DNA polymerase mixture, 3 μL of 10× buffer, and 20 ng of DNA template [24]. PCR products were purified using Multiscreen PCR plates (Millipore). Five microliters of PCR product was mixed with 3.4 μL of H₂O and restricted with 3 U each of Rsal and *Hae*III for ≥30-min incubation at 37°C [24]. The restriction digests underwent electrophoresis for 2.5 h at 150 V through 4% NuSieve 3:1 agarose gels and captured with GelDoc 2000 (Bio-Rad). Profile numbers 1–4 were assigned on the basis of the presence of 2–3 bands between 300 and 1000 bp in size.

**MLST DNA sequencing.** MLST was done using internal fragments of the following 7 housekeeping genes (protein products are shown in parentheses): *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase), and *ddl* (d-alanine-d-alanine ligase), as described at the MLST Web site (available at: http://www.mlst.net). The primer sets were elongated for all genes except *gki*, as described elsewhere [31]. The internal fragments were amplified by PCR using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and run on a 3700 DNA sequencer (Applied Biosystems). Both the for-
Table 1. Distribution of pneumococcal serogroups and penicillin susceptibility patterns among strains causing acute otitis media in 1999 and 2001.

<table>
<thead>
<tr>
<th>Year, susceptibility</th>
<th>Vaccine serogroup</th>
<th>Total (row %)</th>
<th>Non–PNC7 vaccine serogroup</th>
<th>Total (col %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 6 9 14 18 19 23</td>
<td></td>
<td>3 7 11 15 16 20 22 35 NT</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1999</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5 1 1 3 1 11</td>
<td>(91.7)</td>
<td>1</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>R</td>
<td>3 4 5 4 8 24</td>
<td>(88.9)</td>
<td>2 1</td>
<td>3 (11.1)</td>
</tr>
<tr>
<td>S</td>
<td>4 12 1 6 2 6 9 40</td>
<td>(81.6)</td>
<td>3 3</td>
<td>1 (18.4)</td>
</tr>
<tr>
<td>Total</td>
<td>4 20 6 12 2 13 18</td>
<td>75 (85.2)</td>
<td>3 3</td>
<td>13 (14.8)</td>
</tr>
<tr>
<td>2001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>3 1 4 (57.1)</td>
<td></td>
<td>1</td>
<td>2 (42.9)</td>
</tr>
<tr>
<td>R</td>
<td>1 4 14 3 22</td>
<td>(91.7)</td>
<td>1</td>
<td>2 (8.3)</td>
</tr>
<tr>
<td>S</td>
<td>2 4 1 1 4 2 14</td>
<td>(43.7)</td>
<td>5 2 1</td>
<td>18 (56.3)</td>
</tr>
<tr>
<td>Total</td>
<td>2 7 1 5 1 19 5 40</td>
<td>63.5)</td>
<td>5 2 1</td>
<td>23 (36.5)</td>
</tr>
<tr>
<td></td>
<td>6 27 7 17 3 32 23</td>
<td>115 (76.2)</td>
<td>8 2 4</td>
<td>36 (23.8)</td>
</tr>
</tbody>
</table>

NOTE. I, intermediate; PNC7, pneumococcal 7-valent protein conjugate; R, resistant; S, sensitive.

ward and reverse strands were sequenced. Alleles were assigned through the MLST Web site.

RESULTS

A total of 151 middle-ear isolates were obtained from 140 children; 11 children had 2 episodes each of AOM. Data regarding race and sex were available for 97.1% (136/140) of the children; 80.7% (113/136) were white, 13.2% (18/136) were black, and 59.6% (81/136) were male. Using the first episode as the source of information, 97.1% (136/140) of the cases had antibiotic data available. Overall, 59.0% (46/78) of the children who reported recent antibiotic use were infected with a penicillin-nonsusceptible strain, compared with 28.6% (16/56) of the children who did not report recent antibiotic use (P < .01). In 1999, 55.8% (24/43) of the children who reported recent antibiotic use were infected with a penicillin-nonsusceptible strain, compared with 33.3% (12/36) of the children who did not report recent antibiotic use (P = .05). In 2001, 62.9% (22/35) of the children who reported recent antibiotic use were infected with a penicillin-nonsusceptible strain, compared with 20.0% (4/20) of the children who did not report recent antibiotic use (P < .01). Among the 11 children with recurrent AOM, 7 episodes (63.6%) were due to relapses or persistent infections and 4 episodes (36.6%) were due to reinfections. The median interval between persistent disease or relapse was 2.3 weeks (range, 0.3–21.1 weeks). The median interval between reinfections was 12.1 weeks (range, 3.6–30 weeks). The interval between episodes was ≤3 weeks for 85.7% (6/7) of the relapses and >3 weeks for all 4 of the reinfections. In each instance, the pair of sequential isolates from an individual child was not associated with capsular switching.

The remainder of the analysis included all 151 strains. In 1999, 44.3% (39/88) of the strains overall and 30.8% (4/13) of the nonvaccine serogroups were penicillin nonsusceptible. In 2001, 49.2% (31/63) of the strains overall and 21.7% (5/23) of the nonvaccine serogroups were penicillin nonsusceptible (table 1). The proportion of nonvaccine serogroups increased from 14.8% (13/88) to 36.5% (23/63) from 1999 to 2001 (P < .01; table 1). None of the children in 1999 and 38.3% (23/60) of the children in 2001 received 1 dose of PNC7. Among children who received ≥2 doses of PNC7 vaccine, 46.7% (7/15) of the children in 2001 received ≥1 dose of PNC7. Among children who received ≥2 doses of PNC7 vaccine, 46.7% (7/15) of the middle-ear isolates were of nonvaccine serogroups, compared with 20.8% (6/29) of the children who did not receive any doses of PNC7 vaccine (P = .05; table 2).

When PFGE was used as the screening method to detect possible capsular switching, strains from the following serogroups were genetically related: 6-19, 6-14-35, 15-19, 6-18, and Spanish 23F-19. Except for the 6-18 serogroup strains, all of the strains within a capsular switch had identical sequence types (STs) or were single-locus variants (SLVs), according to the results of MLST. The serogroup 6 strain differed from the 2 serogroup 18 strains at all loci except for recP; therefore, they were not classified as capsular switches. During the study, we detected a new ST, which has been designated ST-672 by the MLST Web site (figure 1). All 4 strains that were either identical or were SLVs of ST-672 had gki allele 72, which we submitted as a new allele.

In 1999, 30.8% (4/13) of the nonvaccine serogroups were implicated in capsular switching (strain number in parentheses if >1): 15 and 35 (3). Likewise, in 2001, 26.1% (6/23) of the nonvaccine serogroups were implicated in capsular switching: 15 (4), 35, and 1 that was not typeable (figure 1). The remainder of the nonvaccine serogroup strains were not genetically related to other serogroups. Among vaccine types, serogroups 19 and
DISCUSSION

In the present study, we found that the serogroup distribution of pneumococcal strains causing AOM in children was significantly changed after the licensure of the PNC7 vaccine. The proportion of nonvaccine pneumococcal strains increased over time and among those who received at least 2 doses of the PNC7 vaccine. Among the vaccine types causing AOM in children who received at least 2 doses, serogroup 6 and 19 infections accounted for a large proportion of infections.

Several hypotheses may explain the predominance of these 2 vaccine types. It is possible that these serogroups will be less likely to cause infections after 4 doses of the vaccine [4]. It is also conceivable that the majority of these strains were vaccine related rather than vaccine types, because serotype data were not included. However, the most likely explanation is that this vaccine is less effective for some serotypes than for others. In the Finnish Otitis Media Vaccine Trial, vaccine efficacy against serotype 19F was markedly lower than that against other vaccine serotypes [4]. Moreover, in the randomized control trial that compared the 4-dose regimen of PNC7 with meningococcal vaccine in healthy infants, the single vaccine failure was due to a serotype 19F strain [5]. In a human immunodeficiency virus–seropositive child who received 4 doses of the PNC7 vaccine, recurrent serotype 6B pneumococcal infections were documented [32]. Serotype-specific immunological responses have been shown to be greater for serotype 23F than for serotypes 6B or 19F after AOM infections caused by these serotypes [33].

Because antibiotic treatment does not eradicate colonization, it is not surprising that recurrent disease occurred relatively frequently in our cohort. A cutoff of 3 weeks accurately predicted all reinfections and the majority of the relapses or persistent infections. As has been shown elsewhere, recent antibiotic use was associated with penicillin resistance [3]. Fortunately, the proportion of penicillin-non-susceptible strains from nonvaccine serogroups did not increase over time; however, the presence of the penicillin-non-susceptible serogroup 35B clone [27] underscores the importance of the continued surveillance of the molecular subtypes causing pneumococcal disease during the PNC7 vaccine era.

The present article represents the first comparison of the molecular epidemiology of isolates of S. pneumoniae from episodes of AOM in a large group of children before and after licensure of the PNC7 vaccine. The use of 2 molecular subtyping methods improved our ability to discern which strains had undergone capsular transformation. MLST has been shown to correlate with PFGE [34], and both are typically used to fully characterize drug-resistant international clones, including the Spanish 23F-1 clone [26] and the serotype 35B clone [27]. Because the sequencing of 7 loci using MLST is both expensive and time consuming, PFGE was used for screening, and MLST was used for the confirmation of serotype capsular transformation. Although a PFGE-based cluster implicated serogroup 18 and 6 strains, MLST demonstrated little genetic relatedness between the strains from these serogroups. Because PFGE defines genetic relatedness through band detection, it is conceivable that a similarity in banding patterns is occasionally due to artifact. The restriction profiles of the pbp2b gene provided additional evidence of the genetic relatedness between strains that had undergone capsular switching, because implicated strains had a similar profile when the penicillin susceptibility pattern correlated.

Capsular switching was relatively common but did not account for the total increase in the strains from non–PNC7 vaccine serogroups. Strains from serogroups 35 and 15 accounted for most of the nonvaccine groups that were associated with capsular transformation. The serogroup 35 strains were SLVs of the penicillin-non-susceptible 35B clone [27].

Table 2. Serogroup distribution among those who received 0–4 doses of PNC7 vaccine.

<table>
<thead>
<tr>
<th>No. of doses</th>
<th>Vaccine serogroup</th>
<th>Nonvaccine serogroup</th>
<th>Overall total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>≥1</td>
<td>1</td>
<td>3</td>
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</tr>
<tr>
<td>≥2</td>
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</tr>
<tr>
<td>≥3</td>
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<td>3</td>
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<tr>
<td>≥4</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**NOTE.** Data are expressed as no. (%). NT, not typeable.
35B, unlike serotype 15B, is not represented in the 23-valent pneumococcal polysaccharide vaccine. In our study, serogroup 19 variants of the Spanish 23F clone were frequently isolated, especially from samples obtained during 2001. Continued surveillance of the serogroups and molecular subtypes causing AOM will be of great interest as PNC7 is more widely distributed. These types of studies are important for the development of future formulations of the pneumococcal vaccines.

Acknowledgments

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References