Emergence of a Unique Penicillin-Resistant Streptococcus pneumoniae Serogroup 35 Strain

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We analyzed seven Streptococcus pneumoniae serogroup 35 isolates by pulsed-field gel electrophoresis of the genome and pbp2b gene nucleotide sequences. Three penicillin-susceptible strains and one penicillin-intermediate-resistant strain exhibited 100% identity to prototype R6. Two resistant strains and one other intermediate strain differed from them and contained a unique sequence.

Penicillin-nonsusceptible Streptococcus pneumoniae (PNSP) remains a major global health threat because S. pneumoniae is the predominant bacterial pathogen of community-acquired pneumonia and a major cause of meningitis and of otitis media in children (1, 2, 8, 9, 17, 20, 22, 23, 26, 27, 32, 33, 35, 37). S. pneumoniae develops penicillin resistance through recombination with other penicillin-resistant S. pneumoniae (PRSP) strains or closely related streptococcal species that alter its penicillin binding protein (ppb) genes pbp1a, pbp2a, and pbp2x (5, 10, 13, 19). These altered pbp gene sequences of PNSP differ greatly within the PNSP subset and from the sequences of penicillin-susceptible S. pneumoniae (PSSP) (4, 5, 11, 12, 13, 19, 34, 38, 40).

Serogroups/types (SGTs) 6, 9, 14, 19, and 23 are the most common SGTs associated with penicillin resistance in the United States (15, 39). PNSP strains of other SGTs have been recognized worldwide, especially serotype 35B (7, 15, 21, 25, 28, 29). Between 1983 and 2005, we recovered seven SGT 35 isolates, three of which were PNSP, and other than the five common SGTs, they represented the only other PNSP SGTs in our community (30, 36). Because serotype 35B emerged as a new resistant serotype, we investigated the clonal relationships between SGT 35 PSSP and PNSP strains by pulsed-field gel electrophoresis (PFGE) of the complete genome and pbp2b gene DNA sequences.

**Strains.** Seven SGT 35 strains (0.94%) were identified from a total of 741 invasive S. pneumoniae strains recovered between 1983 and 2005 from adults. Capsular type was determined by include their corresponding amino acids, which were analyzed using the BLASTn program.

DNA sequencing of the pbp2b gene. Six primers were used for DNA sequencing, the forward and reverse primers (see above) with 2 internal forward primers (5’TGCCTTTTGTCTTAGTCCAGG3’ and 5’ACCTTTGTTGAATGTGGTTGG3’ and 2 internal reverse primers (5’AGTTATCAACTGCCAAAGG3’ and 5’ACAAGACTTCATTTCCCAAC3’) that we designed using SeqWeb. Dye terminator sequencing and analysis were completed using an ABI 3130 genetic analyzer. DNA sequences were aligned using multiple-sequence alignment by ClustalW version 1.83 (http://clustalw.genome.jp/). We compared for sequence identity our seven strains to each other, to the susceptible reference strain R6 (GenBank accession no. X16022), and to other strains registered with PubMed using the BLASTn program from NCBI (http://www.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were translated into their corresponding amino acids, which were analyzed using the BLASTn program.

**Probe analysis of the pbp2b gene.** Strains were analyzed for the presence of the five oligonucleotide probes described by Dowson et al. (13). Our strains contained only three of the five probes, namely, probe 1 from PSSP R6 (nucleotides 694 to 714), probe 2 from the PRSP DN87/577 strain (nucleotides 694 to 714), and probe 3 from penicillin-resistant Streptococcus oralis strains 5296 and 5302 (nucleotides 754 to 783). The inclusion of either probe 2 or probe 3 conferred nonsusceptibility (13).

**Comment.** Of our seven SGT 35 strains, three PSSP strains (S0087 [35F], S0453 [35B], and S0980 [35F]) and one low-level-penicillin-intermediate S. pneumoniae (PISP) strain (S0398 [35F]), designated the SUSC group, showed 99% or 100% sequence identity to each other and 99% to R6, differing from R6 by 2 to 7 nucleotides (Table 1). However, there were no differences in amino acids between the SUSC group and R6 as
a result of these nucleotide changes. Two PRSP strains (S1023 [35B] and S1044 [35B]) and one high-level-PISP strain (S1070 [35B]), designated the RESIST group, showed 100% sequence identity to each other (Table 1) and 92% to R6, differing by 115 nucleotides, which resulted in 20 amino acid differences from R6 (Table 1).

The RESIST-group strains were susceptible to tetracycline, trimethoprim-sulfamethoxazole, ofloxacin, and chloramphenicol, with the exception of the PISP strain S1070, which was resistant to tetracycline. These strains were resistant to erythromycin and cefuroxime and intermediate resistant to imipenem and cefotaxime, with the exceptions of S1044 and S1023, which were susceptible to cefotaxime. Strain S0398, which exhibited low-level-intermediate resistance to penicillin, was susceptible to all other antibiotics tested.

Strains of the SUSC group showed two common HaeIII restriction sites, and the RESIST group showed these two sites and two additional common restriction sites (Fig. 1a). Seven HinFI restriction sites were common to the SUSC group, and eight HinFI restriction sites were common to RESIST group strains (Fig. 1b). Five of the HinFI restriction sites occurred in all seven strains, one of which was located in the forward primer. The SUSC group HinFI digestion resolved into four single bands and one double band, which was composed of 78- and 82-bp fragments; two bands smaller than 50 bp could not be identified. HinFI digestion of the RESIST group showed five bands, and four bands, each smaller than 50 bp, could not be identified (Fig. 1b).

By BLASTn analyses, the pbp2b gene DNA sequence of the SUSC group strains showed 99% or 100% sequence identity to several strains in the PubMed database (12, 14, 16, 18). The RESIST group showed 96% sequence identity with two strains registered in PubMed, SP00080 and SP00081 (31). When amino acid sequences were compared, the RESIST group strains showed 99% identity to SP00080, 98% identity to SP00081, and 99% identity to unregistered strains J1 and J88 (4).

Of the three oligonucleotide probes identified in our pbp2b genes, probe 1 was present only in SUSC group strains (4, 11,

### Table 1. Relatedness by nucleotide sequence of the pbp2b gene of penicillin-susceptible and -resistant *Streptococcus pneumoniae* serogroup 35 strains

<table>
<thead>
<tr>
<th>Strain (type)</th>
<th>Penicillin susceptibility level</th>
<th>MIC (µg/ml)</th>
<th>% relatedness to indicated strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6*</td>
<td>Susceptible</td>
<td></td>
<td>R6</td>
</tr>
<tr>
<td>S0087 (35F)</td>
<td>Susceptible</td>
<td>0.032</td>
<td>100</td>
</tr>
<tr>
<td>S0453 (35B)</td>
<td>Susceptible</td>
<td>0.047</td>
<td>100</td>
</tr>
<tr>
<td>S0980 (35F)</td>
<td>Susceptible</td>
<td>0.032</td>
<td>100</td>
</tr>
<tr>
<td>S0398 (35F)</td>
<td>Intermediate</td>
<td>0.094</td>
<td>100</td>
</tr>
<tr>
<td>S1070 (35B)</td>
<td>Intermediate</td>
<td>1.000</td>
<td>100</td>
</tr>
<tr>
<td>S1044 (35B)</td>
<td>Resistant</td>
<td>2.000</td>
<td>100</td>
</tr>
<tr>
<td>S1023 (35B)</td>
<td>Resistant</td>
<td>2.000</td>
<td>100</td>
</tr>
</tbody>
</table>

*GenBank accession no. X16022.

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FIG. 1. (a) This RFLP gel shows two distinct HaeIII patterns of the *pbp2b* gene for seven strains of *S. pneumoniae* SGT 35. Four SUSC group strains (lanes 1 to 4) show one pattern of the *pbp2b* gene (lane 1, strain S0087 [MIC = 0.032 µg/ml]; lane 2, strain S0453 [MIC = 0.047 µg/ml]; lane 3, strain S0980 [MIC = 0.032 µg/ml]; and lane 4, strain S0398 [MIC = 0.094 µg/ml]). Three RESIST group strains (lanes 5 to 7) show a second pattern (lane 5, strain S1070 [MIC = 1.000 µg/ml]; lane 6, strain S1044 [MIC = 2.000 µg/ml]; and lane 7, strain S1023 [MIC = 2.000 µg/ml]). The lanes marked with “m” contain a 100-bp DNA ladder (Invitrogen), with the 600-bp band 2 to 3 times brighter than the other bands, providing internal orientation. (b) This RFLP gel shows two distinct HinFI patterns of the *pbp2b* gene, one for the four SUSC group strains and one for the three RESIST group strains of *S. pneumoniae* SGT 35. All strains are in the same order as in panel a.
13, 23, 38). Probes 2 and 3 were present only in RESIST group strains, both within a resistant block (618 to 891 bp), that contained 57 bp and 11 amino acid substitutions and resulted in 21% divergence from R6 (Fig. 2) (13). The RESIST group strains showed 100% sequence identity in this resistant region to the PRSP SP00091, S. oralis 5296, and Streptococcus mitis B6 strains and 99% sequence identity to Streptococcus sanguis strain 1907, penicillin-resistant S. mitis strain B6, and penicillin-resistant S. oralis strain 5296.

The \( \text{pbp2b} \) gene in the RESIST group strains contained a second resistant block that was not associated with PRSP probe 2. This second resistant nucleotide block, comprising nucleotides 1227 to 1458, diverged from R6 by 13.4% and represents a unique region not registered in the PubMed database. The two sensitive blocks, comprising nucleotides 1 to 617 and 892 to 1226, diverged from R6 by 3.2% and 2.1%, respectively. Nucleotides 1 to 1226 showed 98% sequence identity to several strains registered in the PubMed database, including DN87/577, but the complete 1.5-kb sequence showed a maximum identity of 96% to any strain registered in PubMed because of the unique resistant block comprising nucleotides 1227 to 1458, which showed no more than 86% identity to any registered sequence (Table 2).

The \( \text{pbp2b} \) gene sequences of the strains in the SUSC group were essentially indistinguishable from those of the reference PSSP strain R6 and the serotype 35N strain 2282 (GenBank accession no. DQ071921), identified by Granger et al. (18). The PFGE analysis of the SUSC group showed that the three serotype 35F strains shared the same PFGE profile, which differed from the profile of the single serotype 35B strain (Fig. 3).

### TABLE 2. Deduced amino acid sequences of penicillin-resistant *Streptococcus pneumoniae* serogroup 35 strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Penicillin susceptibility level</th>
<th>Amino acid corresponding to indicated codon&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>R6</td>
<td>Susceptible</td>
<td>V A E I S N T Q Q T L S E S T N D Q T Q K Y</td>
</tr>
<tr>
<td>S1070</td>
<td>Intermediate</td>
<td>G L P Y K L E A I T G A A D G E N N Q H</td>
</tr>
<tr>
<td>S1044, S1023</td>
<td>Resistant</td>
<td>G L P Y K L E A I T G A A D G E N N Q H</td>
</tr>
<tr>
<td>SP00080 (31)</td>
<td>Resistant</td>
<td>I S G L P Y K L E A I T G A A D G E N N Q H</td>
</tr>
<tr>
<td>J1 (4)</td>
<td>Intermediate</td>
<td>G L Y K L E A I T G A A D G E N N Q H</td>
</tr>
<tr>
<td>J88 (4)</td>
<td>Intermediate</td>
<td>G L Y K L E A I T G A A D G E N N Q H</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only amino acids that differ from those of the PSSP strain R6 are shown; a dash indicates an amino acid identical to that of R6.
We do not have any association that might pose a conflict of interest.

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