Emergence of a Novel Penicillin-Nonsusceptible, Invasive Serotype 35B Clone of \textit{Streptococcus pneumoniae} within the United States

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Monitoring antibiotic-resistant pneumococcal strains not covered by the 7-valent conjugate vaccine is an important priority. The Centers for Disease Control and Prevention’s Active Bacterial Core Surveillance identified 68 invasive penicillin-nonsusceptible serotype 35B (PN35B) isolates recovered from 1995 to 2001 from patients residing in the states of California, Colorado, Connecticut, Georgia, Maryland, Minnesota, New York, Oregon, Tennessee, and Texas. Nonsusceptible isolates accounted for 69% of all serotype 35B isolates recovered during this time. Twelve (18%) of the 68 PN35B isolates recovered since 1995 were obtained from pediatric patients. These 68 isolates exhibited penicillin MICs of 0.25–2 μg/mL and reduced susceptibility to cefotaxime. Representative PN35B isolates exhibited a common chromosomal macrorestriction profile and identical penicillin-binding–protein gene restriction profiles characteristic of penicillin-resistant strains, and they shared a unique 7-locus sequence type that included 3 new alleles. The mosaic \textit{pdb} 2b and divergent \textit{dd}l sequences were suggestive of interspecies recombination at the \textit{dd}l-\textit{pdb} 2b chromosomal region.

There has been a rapid increase in the proportion of penicillin-resistant pneumococci in the United States in recent years [1]. Whereas many prevalent penicillin-resistant strains appear to have originated in countries other than the United States, certain new resistant strains were first identified within the United States [2–4].

\(\beta\)-Lactam resistance in pneumococci requires the development of altered penicillin-binding proteins (PBPs) that bind \(\beta\)-lactams with lower affinity [5]. Third-generation cephalosporin resistance can be conferred by changes within the closely linked \textit{pdb} 1a and \textit{pdb} 2x genes [6], whereas penicillin resistance also requires alteration of the \textit{pdb} 2b gene [7]. Analysis of PBP genes from resistant isolates indicates that recombination events with closely related streptococcal species have been necessary for the development of \(\beta\)-lactam resistance in pneumococci [8].

The majority of penicillin resistance occurs within serogroups 6, 9, 14, 19, and 23, which commonly infect young children [1, 6]. At the current time, penicillin resistance is rare or is as yet undetected among many serotypes more frequently recovered from adults [1]. Serotype 35B is among the serotypes that are not often associated with disease in young children and are not included in the 7-valent conjugate vaccine. Here we report the molecular typing of an invasive penicillin-nonsusceptible serotype 35B (PN35B) clone that has been recovered through the Centers for Disease Control and Prevention’s Active Bacterial Core Surveillance (ABCs)/Emerging Infections Program Network. This strain has caused invasive disease in pediatric and adult patients from 10 different states within the United States during the past 6 years.

Materials and Methods.

\textbf{Isolates.} All isolates were obtained from sterile-site specimens through the ABCs [9]. Isolates were serotyped by latex agglutination, and serotype was confirmed by positive Quellung reactions. Antibiotic MICs were determined by the broth microdilution system, according to procedures of the National Committee for Clinical Laboratory Standards [3]. Pulsed-field gel electrophoresis (PFGE) was performed as described elsewhere [4]. Sixteen internationally dispersed antibiotic-resistant strains [10] were used for PFGE comparisons.

\textbf{Restriction-enzyme profiling.} PBP gene amplicons were subjected to \textit{Hae} III plus \textit{Rsa} I digest analysis, as described elsewhere [3], except that 4% NuSieve 3:1 gels were used.
DNA sequencing. Multilocus sequence serotypes were determined, as described elsewhere [11], by use of the Multilocus Sequence Typing Web site (http://www.mlst.net). Isolates that share a ≤1-allele difference within the 7 alleles that constitute a multilocus sequence type (MLST) are indicative of the same clone, and isolates with <4 identical alleles do not share a high degree of genetic relatedness [12]. Sequencing of a 650-bp php2b amplicon fragment was performed as described elsewhere [13].

Results

Appearance of PN35B isolates within the United States. In brief, ABCs is an active, population-based surveillance system that operated in 10 US states between 1995 and 2001. During 1995–2001, we identified 99 serotype 35B sterile-site isolates from ABCs sites (see http://www.cdc.gov/ncidod/dbmd/abcs/ for information concerning ABCs surveillance population), of which 68 (69%) were resistant or intermediately resistant to penicillin (table 1). All 68 PN35B isolates showed decreased susceptibility (MIC, 0.25–0.5 μg/mL) or nonsusceptibility (MIC, ≥1 μg/mL) to cefotaxime. Except for instances of trimethoprim-sulfamethoxazole resistance (11 isolates [16%]), tetracycline resistance (3 isolates [4%]), and levofloxacin resistance (2 isolates [3%]), there were no other resistances seen among 67 of the 68 PN35B isolates. One penicillin-resistant isolate was highly resistant to cefotaxime (MIC, 8 μg/mL) and was also resistant to trimethoprim-sulfamethoxazole, tetracycline, erythromycin, clindamycin, and chloramphenicol.

Thirty-five (51%) of the 68 PN35B isolates were recovered from individuals >60 years of age; however, 12 (18%) were recovered from pediatric patients. At least 1 isolate was found in each of the 10 surveillance areas.

PN35B isolates constitute a single unique clone. PFGE analysis revealed that all the PN35B isolates available (5 [8%] of the 68 isolates were not retrievable) shared an identical or closely related profile (representative isolates depicted in figure 1A). This profile was also exhibited by the single penicillin-resistant isolate described above that was resistant to cefotaxime and to 6 other antibiotics.

PFGE analysis revealed that 26 (84%) of the 31 penicillin-sensitive serotype 35B (PS35B) pneumococcal isolates analyzed shared an identical or closely related profile, and only 1 of the profiles from PS35B isolates bore any resemblance to the profile shared by the PN35B isolates (representative profiles from PS35B and PN35B isolates are shown in figure 1A). In addition, the PN35B isolate PFGE profile was not closely related to the profiles in our database obtained from >1200 invasive isolates and 16 internationally dispersed antibiotic-resistant clones [10]. However, PS35B invasive isolate 926-01, recovered from a patient in New York in 2000, did share the conserved PN35B profile (data not shown). Unlike the other 30 PS35B isolates recovered during this period, which had penicillin MICs ≤0.03 μg/mL, this isolate had a MIC of 0.06 μg/mL.

The same MLST was obtained from 2 independent PN35B isolates obtained in 1999 (isolates 1894-00 and 6361-99 from New York and Tennessee, respectively) that shared the common PN35B PFGE profile. In addition, this MLST was shared by the single penicillin-sensitive isolate with a penicillin MIC of 0.06 μg/mL described above (New York isolate 926-01). The PN35B MLST (aroE18, gdh12, gki4, recP44, spi14, xpt77, and ddl82) was unique and shared only 2 alleles with the 2 closest MLSTs in the extensive database at the Multilocus Sequence Typing Web site (http://www.mlst.net; aroE18 and gki4 from a serotype 19F strain and gdh12 and gki4 from a serotype 14 strain). A further indication of the genetic uniqueness of this clone was the observation that 3 of these alleles (recP44, xpt77, and ddl82) did not exist in the MLST database prior to our submission of these sequences. The MLST obtained from a single PS35B isolate representative of the major PS35B PFGE profile (figure 1A) shared only 1 common allele (spi14) with the PN35B clone MLST (aroE7, gdh9, gki9, recP1, spi14, xpt48, and ddl14, compared with aroE18, gdh12, gki4, recP44, spi14, xpt77, and ddl82).

The PN35B clone has PBP gene restriction profiles and a

Table 1. Pneumococcal isolates of a penicillin-nonsusceptible type 35B (PN35B) clone recovered from Active Bacterial Core Surveillance (ABCs) during 1995–2001.

<table>
<thead>
<tr>
<th>Year</th>
<th>Penicillin MIC</th>
<th>Age of patient, years</th>
<th>State PN35B isolated (no. of isolates) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25</td>
<td>0.5–1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>1995</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>1996</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1997</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1998</td>
<td>0</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>2000</td>
<td>2</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>


a States are indicated by standard 2-letter abbreviations.

b Data for 2001 are incomplete.
Figure 1.  A. Pulsed-field gel electrophoresis (PFGE) profiles of chromosomal \textit{Sma}I digests representative of 13 penicillin-sensitive serotype 35B (PS35B; \textit{lanes} 1–14) and 14 penicillin-nonsusceptible serotype 35B (PN35B; \textit{lanes} 15–27) isolates recovered during 1995–2001. I, R, and S indicate intermediate penicillin resistance, penicillin resistance, and penicillin sensitivity, respectively. States where isolates were recovered are indicated by standard 2-letter abbreviations, and year of isolation for individual isolates are indicated.

B. Representative penicillin-binding protein gene amplicons from PN35B isolates digested with \textit{Hae}III/\textit{Rsa}I. \textit{Lanes} 2–7, 8–13, and 14–19 depict \textit{pbp1a}, \textit{pbp2b}, and \textit{pbp2x} restriction profiles, respectively. These same isolates (\textit{lanes} 2–6, 8–12, and 14–18) were among those used to depict the PN35B PFGE profile. In addition, penicillin-binding–protein gene \textit{Hae}III + \textit{Rsa}I restriction profiles commonly associated with penicillin sensitivity (profiles \textit{pbp1a}-1, \textit{pbp2b}-1, and \textit{pbp2x}-1 described in [3]) are shown in \textit{lanes} 7, 13, and 19 from a single penicillin-sensitive serotype 6B strain.

\textbf{pbp2b} sequence characteristic of \textit{b-lactam–resistant} strains. \textit{b-lactam–sensitive} pneumococcal strains display very little sequence variation between their PBP genes, which is generally shown by highly conserved PBP gene restriction profiles. Restriction-digest profiles of \textit{pbp1a}, \textit{pbp2b}, and \textit{pbp2x} amplicons revealed conserved profiles among the PN35B isolates (figure 1B). This \textit{pbp1a} profile was different from all previous \textit{pbp1a} profiles that we have examined, whereas the \textit{pbp2b} and \textit{pbp2x} profiles were indistinguishable from profiles \textit{pbp2b}-14 and \textit{pbp2x}-2 (designations assigned in [3]). Profile \textit{pbp2x}-2 has been observed previously in many multiresistant isolates of serogroups 6, 9, 19, 14, and 23, including the well-characterized strains Spain$^{\text{3F}}$-1 and France$^{\text{9F}}$-3 [3].

It was interesting to note that profile \textit{pbp2b}-14 was previously found in a single \textit{b-lactam–resistant} serotype 6B isolate [3]. We also observed that a 650-bp \textit{pbp2b} amplicon sequence from the PN35B clone isolates 1894-00 and 6361-99 was nearly identical (657 and 659 bp, respectively) to the corresponding sequence of a \textit{pbp2b} allele from a penicillin-resistant serotype 6 isolate recovered from a patient in South Africa (GenBank accession no. U20073, 370–1028 bp) [14] and that the deduced amino acid sequences were identical. The central portion of the transpeptidase-encoding section of these 2 \textit{pbp2b} alleles encodes an alanine substitution at residue Thr-252 and a glycine substitution at Glu-282 that are apparently critical for decreased affinity of Pbp2b for penicillin [14]. This segment of these \textit{pbp2b} alleles shares only ~88% sequence identity with the highly conserved \textit{pbp2b} sequences found among penicillin-sensitive pneu-
mococcal strains. It is known that \textit{ddl}, one of the alleles used to determine MLST, lies only 783 bp downstream of \textit{pbp2} on the pneumococcal chromosome [15]. The 441-bp \textit{ddl} gene segment from the PN35B clone (\textit{ddl}82) was more similar to \textit{ddl} alleles from resistant pneumococci and \textit{Streptococcus mitis} (96.1\% identical to \textit{ddl}12 and 93.4\% to \textit{ddl}2 from \textit{S. mitis}) than to the closest matching \textit{ddl} allele from sensitive pneumococci (92\% identity to \textit{ddl}2), which is highly suggestive of past interspecies recombination at the \textit{pbp2b-ddl} chromosomal region [15]. It was interesting to note that this resistant \textit{pbp2b} allele sequence and the divergent \textit{ddl}82 allele sequence were also found in isolate 926-01 (the single PS35B isolate that shared the PN35B PFGE serotype and MLST). Restriction-proﬁle analysis of \textit{pbp1a}, \textit{pbp2b}, and \textit{pbp2x} from 926-01 revealed identity to the PN35B PBP gene proﬁles (ﬁgure 1B), except for a slight difference in \textit{pbp2x} (data not shown). The genetic basis of the rather extensive range of penicillin MIC variation (0.06–2.0 \text{µg/mL}), observed among this highly genetically related set of 69 serotype 35B isolates, remains to be determined.

**Discussion**

Over the past 20 years, pneumococci have displayed an amazing capacity to respond to antibiotic selective pressure through natural mechanisms of uptake and genomic integration of DNA from closely related strains in their environment. Penicillin resistance is predominantly found among pneumococci of serotypes that are commonly recovered from young children and are included in the 7-valent conjugate vaccine [1]. It is unknown whether the restriction of penicillin resistance to certain serotypes is due to innate biological features of certain strains or to the high usage of \textit{β}-lactam antibiotics among pediatric patients. The occurrence of a resistant clone of serotype 35B, which is a serotype more associated with adult disease, is reason for concern. We now have detected this PN35B clone in all 10 sites that have participated in the ABCs system. This strain has caused invasive disease in 12 children <6 years of age in our surveillance population during 1995–2000.

Pneumococcal clones frequently take up DNA in order to change the serotype of their antiphagocytic capsule. Therefore, we thought that the PN35B clone might have genomic relatedness to previously characterized penicillin-resistant strains. Instead, we found that it was a totally novel clone characterized by 3 newly encountered MLST alleles. The appearance and apparent geographic expansion of the PN35B clone over the past 6 years indicates that it is a biologically fit strain. Although table 1 shows an increase of the number of PN35B clone isolates each year since 1995, it is possible that this increase may be due to modiﬁcations in the surveillance system. The system went from serotyping only some of the surveillance isolates during 1995 to serotyping all of the isolates since 1998, and, also, additional surveillance sites were added during this time. It will be of interest to monitor the frequency of occurrence and distribution of this clone during coming years. Judging from the wide geographic range of the PN35B clone within the United States, we predict that this strain is also disseminated internationally; however, at present, we were unable to find records of PN35B isolates recovered in other countries. Surveillance systems that routinely conduct serotyping should monitor for the presence of this and other antibiotic-resistant clones.

Detection and surveillance of individual clones require the use of precise molecular methods. Continued use of this methodology is critical for monitoring the effects of vaccination on genetic variation in pathogenic pneumococci and for identifying antibiotic-resistance reservoirs. We feel that it is important to provide new antibiotic-resistant strains such as the PN35B clone to the international Pneumococcal Molecular Epidemiology Network [10]. This may help facilitate the speedy identiﬁcation of genotypic traits that allow for the biologic success of a relatively limited number of antibiotic-resistant clones.

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**References**


