Clonal Association between *Streptococcus pneumoniae* Serotype 23A, Circulating within the United States, and an Internationally Dispersed Clone of Serotype 23F

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*Streptococcus pneumoniae* is an important pathogen in the United States and is associated with significant morbidity and mortality. Since the introduction of the seven-valent conjugate vaccine, a significant decline in pneumococcal disease has been reported. However, surveillance for pneumococcal disease remains essential, as the extent of cross protection against vaccine-related serotypes is still unclear. Further, any increase in non-vaccine-related serotypes also needs monitoring. We report on a new clonal association between a vaccine-related serotype, serotype 23A, obtained as part of the Active Bacterial Core surveillance, with an established internationally dispersed Pneumococcal Molecular Epidemiology Network (PMEN) clone, clone Colombia23F-26. Sixty-two isolates of serotype 23A collected from sterile sites during a 2-year period (2002 and 2003) were characterized. Twenty-one (34%) isolates were penicillin nonsusceptible, although none were fully resistant. Pulsed-field gel electrophoresis and multilocus sequence typing analysis showed that 24 (39%) of the serotype 23A isolates shared either genetic identity or high genetic relatedness with PMEN clone Colombia23F-26. Extensive variability was noted within the sequenced region of *pbp2b* in two penicillin-nonsusceptible isolates as well as in PMEN clone Colombia23F-26, suggesting that these isolates probably acquired penicillin resistance independently. The emergence of such new serotype and genotype associations highlights the dynamic nature of the pneumococcal population, necessitating continuous monitoring in the post-vaccine era.

*Streptococcus pneumoniae* is a major cause of morbidity and mortality in the United States, with the highest rates of disease seen among young children and elderly individuals (5, 22). In the United States most deaths from pneumococcal disease occur among elderly individuals (5). However, pneumococcal disease is also responsible for significant morbidity in the pediatric population. Prior to the introduction of conjugate pneumococcal vaccine, a high rate (166.9/100,000 population) of invasive pneumococcal disease was seen among children less than 2 years of age.

The first pneumococcal conjugate vaccine was licensed for use in infants and young children in the United States in 2000 (25). During a controlled clinical trial before licensure, this seven-valent vaccine showed excellent efficacy (97.4%) against invasive disease caused by all seven serotypes targeted in the vaccine (2), with a significant impact on otitis media and pneumonia. The vaccine was also found to reduce nasopharyngeal carriage of vaccine-type pneumococci (7, 16). However, this decrease was accompanied by a concomitant increase in colonization with non-vaccine-related types (19, 24). More recently, the Centers for Disease Control and Prevention’s (CDC’s) Active Bacterial Core surveillance (ABCs) reported a significant decline in invasive disease caused by the vaccine serotypes in children below 2 years of age (25). The vaccine, however, has been found to have a different efficacy against the different vaccine-related serotypes (10), and the extent of cross protection provided to the vaccine-related serotypes is still unclear. This emphasizes the need for continuous monitoring for pneumococcal disease to document any changes in the serotype pattern and antimicrobial susceptibility profile and to detect the emergence and spread of new clones or new clonal associations, as the use of conjugate vaccine changes the balance of circulating strains.

Here, we report on the emergence of a new association in the United States between multiple isolates of vaccine-related serotype 23A with an established internationally dispersed clone of serotype 23F. All serotype 23A isolates obtained from adult and pediatric patients in eight different states over a 2-year period (2002 and 2003) were characterized. Twenty isolates of serotype 23F obtained during the same time period from the same surveillance areas with various antibiotic susceptibility patterns were also chosen for comparison to determine their relation to Pneumococcal Molecular Epidemiology Network (PMEN) clone Colombia23F-26.

MATERIALS AND METHODS

Cases of invasive pneumococcal disease were identified through ABCs, part of CDC’s Emerging Infections Program. ABCs is an active, population-based, laboratory-based surveillance system that has been continuously monitoring invasive pneumococcal infections since January 1996, with 16 million individuals currently under surveillance (12). Invasive disease was defined by the isolation of pneumococci from a normally sterile site among residents of the surveillance population, as described previously (12). Analysis of trends in the incidence of invasive disease caused by serotype 23A over time was limited to the surveillance counties under continuous surveillance from 1999 to 2003, with the number of serotype 23A isolates identified as the numerator and the U.S. Census estimates for the ABCs population identified as the denominator.

Serotyping was performed by latex agglutination, and the serotype was con-
firmed by Quellung reaction (with sera prepared at CDC). The MIC was determined by the broth microdilution method for the following antimicrobials: penicillin, amoxicillin, cefotaxime, chloramphenicol, tetracycline, clindamyacin, erythromycin, levofloxacin, meropenem, vancomycin, and trimethoprim-sulfamethoxazole. MICs were interpreted by using the CLSI (formerly the National Committee for Clinical Laboratory Standards) guidelines (17). The internationally dispersed clone Colombia23F-26 included for comparison was recovered most of the penicillin-susceptible isolates. Twenty isolates of serotype 23F with a wide range of MICs to penicillin were recovered from patients with meningitis. Of the remaining four isolates (17%), three had a penicillin MIC of 0.06 μg/ml, which is 1 dilution below the breakpoint for intermediate resistance, while one was completely susceptible (0.03 μg/ml). Only one isolate with intermediate resistance to penicillin was not found to show any similarity to this clonal cluster. Except for California, each surveillance site had at least one serotype 23A isolate that showed this genetic association with Colombia23F-26. The serotype 23A isolates that did not show genetic similarity to this clone were divided into several additional lineages and included most of the penicillin-susceptible isolates. Twenty isolates of serotype 23F with a wide range of MICs to penicillin collected from ABCs sites during the same time period were also included for comparison. Only 1 serotype 23F isolate with intermediate resistance to penicillin was found to show 75% homology to this clonal cluster, while the 19 other serotype 23F isolates showed no similarity in their PFGE profile to Colombia23F-26 (all shared <70% PFGE pattern similarity).

The Colombia23F-26 clone and the 24 isolates of serotype 23A sharing highly related PFGE profiles were represented by the same multilocus sequence type (ST; ST338). An ST338 strain expressing serotype 23A has previously been reported only in a single blood isolate recovered in California in 1999 (15). One other serotype 23A isolate in our collection appeared to be an outlier that exhibited about 75% PFGE similarity to Colombia23F-26 and was a single-locus variant (SLV; ST172) of ST338. Note that we have previously found ST172 only among serotype 19A isolates (13), and in the MLST database (www.mlst.net) ST172 is associated with only serotype 19A isolates. The single serotype 23F isolate sharing PFGE similarity with Colombia23F-26 was also ST172. The remaining 62% of the serotype 23A isolates with no genetic relatedness to the ST338 clonal cluster had STs recorded at mlst.net that have been restricted to serotype 23A (ST1338, ST1336, ST42); the single exception was ST1448, which is related only to the ST338 clonal cluster had STs recorded at mlst.net that have been restricted to serotype 23A (ST1338, ST1336, ST42); the single exception was ST1448, which is

<table>
<thead>
<tr>
<th>Yr</th>
<th>No. of isolates</th>
<th>Age (yrs)</th>
<th>No. of isolates with the following penicillin MIC (μg/ml)</th>
<th>Surveillance sites (no. of isolates)*</th>
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<tr>
<td></td>
<td></td>
<td>≤5</td>
<td>6–59</td>
<td>≥60</td>
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<tr>
<td>2002</td>
<td>24</td>
<td>9</td>
<td>15</td>
<td>16</td>
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<tr>
<td>2003</td>
<td>38</td>
<td>2</td>
<td>14</td>
<td>25</td>
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* MN, Minnesota; CA, California; CT, Connecticut; GA, Georgia; MD, Maryland; NY, New York; TN, Tennessee; OR, Oregon.

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<tr>
<th>Age (yrs)</th>
<th>No. of isolates with the following penicillin MIC (μg/ml)</th>
<th>Surveillance sites (no. of isolates)*</th>
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<tr>
<td>≤0.06</td>
<td>8</td>
<td>MN (4), CA (2), CT (7), GA (5), MD (2), NY (2), TN (2)</td>
</tr>
<tr>
<td>0.125–1.0</td>
<td>13</td>
<td>MN (9), CA (2), CT (6), GA (4), MD (5), NY (5), OR (2), TN (5)</td>
</tr>
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Unique association between 23A and PMEN Colombia23F-26. PFGE analysis of the 62 isolates showed that 21 (34%) serotype 23A isolates shared either identical or highly similar (≥80%) banding patterns with the internationally dispersed clone Colombia23F-26 (Fig. 1), with 3 other isolates sharing >75% homology with the cluster and so were considered to belong to the same cluster. Of these 24 isolates, 20 (83%) were ST172. The remaining four isolates (17%), three had a penicillin MIC of 0.06 μg/ml, which is 1 dilution below the breakpoint for intermediate resistance, while one was completely susceptible (0.03 μg/ml). Only one isolate with intermediate resistance to penicillin was not found to show any similarity to this clonal cluster. Except for California, each surveillance site had at least one serotype 23A isolate that showed this genetic association with Colombia23F-26. The serotype 23A isolates that did not show genetic similarity to this clone were divided into several additional lineages and included most of the penicillin-susceptible isolates. Twenty isolates of serotype 23F with a wide range of MICs to penicillin collected from ABCs sites during the same time period were also included for comparison. Only 1 serotype 23F isolate with intermediate resistance to penicillin was found to show 75% homology to this clonal cluster, while the 19 other serotype 23F isolates showed no similarity in their PFGE profile to Colombia23F-26 (all shared <70% PFGE pattern similarity).

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**RESULTS**

Between 1998 and 2003, a significant increase in the rate of disease caused by serotype 23A was observed among older children (age, >5 years) and adults (0.09 cases per 100,000 population in 1998 and 1999 to 0.19 cases per 100,000 population in 2002 and 2003; P = 0.002). However, there was no obvious trend in children <5 years of age (P = 0.560). A total of 62 isolates collected in 2002 and 2003 were characterized as part of this study. Thirty-seven (60%) isolates were from patients ≥60 years of age and two (3%) were from children <5 years of age, with at least two isolates collected from each surveillance site (Table 1). Eleven percent of these isolates were recovered from patients with meningitis.

Most serotype 23A isolates were susceptible to penicillin (66%); 21 isolates (34%) were intermediate resistant to penicillin, and none were fully resistant. All isolates were susceptible to amoxicillin, cefotaxime, meropenem, vancomycin, and levofloxacin. Resistance to most of the other antimicrobials except trimethoprim-sulfamethoxazole (resistance rate, 16%) was uncommon (erythromycin, 3%; clindamyacin, 3%; tetracycline, 3%; chloramphenicol, 1.6%). Two type 23A isolates (3%) were multidrug resistant (resistant to three or more classes of antimicrobials).
netic identity at the PFGE and/or MLST level to previously characterized serotype 23F isolates with STs 713, 714, 81, and 850. The eBURST (http://www.mlst.net) analysis of the STs in the study showed that both ST338 and ST172 are SLVs of ST361 that includes isolates of serotype 23F. Further, eBURST analysis also showed that five of the SLVs originating from ST338 consisted of serotype 23F isolates.

Since the serotype 23A isolates within the ST338 genetic cluster and Colombia23F-26 shared the trait of intermediate penicillin resistance, it was of interest to examine the sequence of a key section of \( pbp2b \) that is associated with resistance. Extensive sequence divergence between Colombia23F-26 and two serotype 23A ST338 isolates were found within a 636-bp region. While all three alleles were clearly mosaic in nature (they included segments of nonpneumococcal sequences), they were also markedly divergent from each other. Two common substitutions associated with penicillin resistance (Thr-252-Ala and Glu-282-Thr) were evident in Colombia23F-26 and one of the serotype 23A isolates. The second 23A isolate examined contained only the Thr-252-Ala substitution. This 636-base \( pbp2b \) segment of all three alleles shared \( 93\% \) identity to the corresponding sequence from susceptible strain R6.

**DISCUSSION**

The prevention of pneumococcal disease is a public health priority for the United States. The objectives outlined in Healthy People 2010 include decreasing the incidence of invasive pneumococcal infections to 46 and 42 cases per 100,000 among children (age, <5 years) and older adults (age, ≥65 years), respectively, by the year 2010 (8). While the introduction of the seven-valent pneumococcal vaccine has helped to achieve this objective in children (12, 25), it has also focused considerable attention on a possible postvaccine scenario with an increase in disease caused by non-vaccine-related serotypes (3, 4). Further, the emergence of new penicillin-nonsusceptible clones with serotypes not covered by the vaccine is alarming (20, 21).

The appearance of a novel clonal association between a vaccine-related serotype 23A and an internationally established clone in the postvaccine era is of particular concern. Colombia23F-26 was first described from penicillin-nonsusceptible invasive isolates recovered in Colombia, Brazil, and Iceland between 1989 and 1996 (23). This association has also been observed in a single serotype 23A blood isolate recovered in Malaysia in 1999 (15). The serotype 23A isolates sharing genetic identity with Colombia23F-26 and one of the serotype 23A isolates recovered in 1999 to be highly related to Colombia23F-26, one of which was from a child with meningitis (data not shown), indicating that the clone probably originated even before the introduction of the vaccine and is perhaps increasing in frequency due to the selection pressure exerted by the vaccine. Also, the increase in serotype 23A and the clone mentioned above is seen mostly among adults and not in children <5 years of age, possibly due to the cross protectivity provided by the seven-valent conjugate vaccine (PCV7).
Capsular transformation, where pneumococci take up and incorporate serotype-specific DNA from other strains, is a well-documented phenomenon (18). Because vaccines induce serotype-specific antibodies, a capsular switch could result in evasion of the host immune response. A sample of serotype 23F isolates collected during the same time period as the serotype 23A study isolates showed one isolate with genetic relatedness to Colombia23F-26, indicating the possibility that the serotype 23A derivative of Colombia23F-26 arose from the existing serotype 23F clone within the United States either by capsular switching or by an intraspecies genetic alteration that resulted in the conversion of serotype 23F to 23A. However, additional comparisons to 123 isolates of serotypes 23F (ABCs) obtained between 1999 and 2001 showed that none were related to the Colombia23F-26 clone (13; unpublished data). Interestingly, the eBURST analysis also showed that most SLVs of ST338 occur within serotype 23F, suggesting that the strains of serotype 23A perhaps originated from serotype 23F, although the evidence presented here may not be adequate to confirm this finding.

β-Lactam resistance in pneumococcal isolates is invariably associated with altered penicillin-binding proteins (PBPs) that bind to β-lactams with a low affinity (14). While approximately two-thirds of the serotype 23A isolates in the study were penicillin susceptible and did not show any relation to the PMEN international clone Colombia23F-26, the strains within the ST338 genetic cluster were intermediately penicillin resistant, suggesting an association between penicillin nonsusceptibility and ST338. Additionally, four of these susceptible isolates within the ST338 cluster had values close to the breakpoint for intermediate resistance. A comparison of the transpeptidase-encoding region of pbp2b also showed high divergence among Colombia23F-26 and the two serotype 23A ST338 cluster isolates examined, suggesting that these isolates may have independently acquired non-pneumococcal pbp2b sequences, which appears to be a prerequisite for penicillin resistance among naturally occurring strains (6, 14). Full penicillin resistance requires multiple genetic alterations within at least three PBP gene targets, and it remains to be seen if these intermediately resistant serotype 23A isolates will accumulate the full complement of changes required for complete resistance to penicillin. For example, such a transition appears to be actively ongoing within the major serotype 19A clone, ST199, which was primarily represented by penicillin-sensitive and intermediately resistant isolates during 1999, with only a few fully resistant isolates (13).

Surveillance and prompt detection of such clonal associations are critical for monitoring the effects of vaccination on the pneumococcal population. It has recently been reported that multiple invasive representatives of a serotype 11A derivative of the highly successful Spain9V-3 (ST156) clone have been detected in Israel (21). It remains to be seen whether this new serotype 11A strain will become a major pathogen. Similarly, it will be of interest to monitor the ability of the serotype 23A equivalent of Colombia23F-26 to become established and disseminated as a biologically successful clone.

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