Molecular Analysis by Pulsed-Field Gel Electrophoresis and Antibiogram of Streptococcus pneumoniae Serotype 6B Isolates from Selected Areas within the United States

KAREN M. RUDOLPH, 1,2 ALAN J. PARKINSON, 1 AND MARILYN C. ROBERTS 2*

Arctic Investigations Program, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Anchorage, Alaska 99508, 1 and Department of Pathobiology, University of Washington, Seattle, Washington 98195 2

Received 10 April 1998/Returned for modification 27 May 1998/Accepted 16 June 1998

Fifty-eight clinical isolates of Streptococcus pneumoniae serotype 6B, including 16 from Alaska, 14 from Arizona, 11 from Washington, and 17 from seven additional states, were analyzed. The antibiograms of these isolates were assigned to 10 antibiotic profiles based on their susceptibilities to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole. Thirty-two (55%) of these isolates were penicillin nonsusceptible, while 21 (36%) were intermediate or resistant to three or more antibiotics. The restriction endonucleases Apal and SmaI were used to digest intact chromosomes, and the fragments were resolved by pulsed-field gel electrophoresis (PFGE). The Apal and SmaI PFGE patterns were combined, and 13 of the 16 Alaskan isolates showed indistinguishable PFGE patterns. One other isolate exhibited highly related Apal and SmaI PFGE patterns, differing by only one band after restriction with Apal. Among the 14 isolates from Arizona, 1 was indistinguishable from the predominant Apal and SmaI PFGE patterns seen in the Alaskan isolates; 5 others were highly related (± 1 band after cutting with either enzyme) to the Alaskan isolates, suggesting a common ancestral origin. Of the remaining eight isolates, six additional Apal plus SmaI PFGE patterns were observed. The 28 isolates from the various contiguous states had 22 Apal plus SmaI PFGE patterns. No correlations were found between specific PFGE patterns, antibiograms, dates of isolation, or geography. The serotype 6B isolates across the contiguous United States were genetically diverse, while the 6B isolates from Alaska appeared to be much less diverse.

Streptococcus pneumoniae remains a leading cause of bacterial pneumonia, otitis media, sepsis, meningitis, and bacteremia worldwide, resulting in significant morbidity and mortality. In the United States it is estimated that S. pneumoniae is responsible for at least one-fourth of all community-acquired pneumonia (6). Over the past decade in the United States, there has been an increase in the number of reports of pneumococcal isolates that are either moderately or completely resistant to penicillin, erythromycin, and trimethoprim-sulfamethoxazole (TMP-SMZ) (28). Isolates resistant to these antibiotics appear to belong to a few selected serogroups and/or serotypes (6B, 14, 19F, 23F, 9V) (1, 11, 14). These serotypes are commonly associated with invasive disease and are most frequently isolated from children with serious infections (4).

In Alaska, the highest reported rate of invasive pneumococcal disease exists among Alaska Natives, specifically Yup’ik Eskimo infants, from the Yukon-Kuskokwim Delta (YKD) region, where in every 40 infants is diagnosed with the invasive disease during their first 2 years of life, a rate of 1,000 per 100,000 per year. This rate is 8 to 10 times higher than for other U.S. population groups (7, 10). Many more cases of pneumococcal disease go undiagnosed because of inaccessibility to culture before empiric antibiotic administration (10). In Alaska since 1986, there has been a 6.5-fold increase in the occurrence of pneumococcal isolates causing invasive disease that have reduced susceptibility to penicillin (MIC ≥ 0.125 μg/ ml), an 8-fold increase in ceftriaxone resistance (MIC ≥ 2 μg/ ml), a 12-fold increase in erythromycin resistance (MIC ≥ 1 μg/ ml), and a 26-fold increase in TMP-SMZ resistance (MICs, ≥ 4 and 76 μg/ml) (24). The highest rates of penicillin-nonsusceptible and multidrug-resistant pneumococci have been found among Alaska Natives of the YKD region of the state. These isolates now represent ≥ 30% of the total S. pneumoniae isolates from the YKD area. In the last few years, these isolates have spread to other areas within Alaska. Of these isolates, a majority (80%) have been identified as serotype 6B (24).

In this study, we utilized pulsed-field gel electrophoresis (PFGE) to analyze susceptible and multidrug-resistant invasive pneumococcal serotype 6B isolates from Alaska and Arizona, where high rates of pneumococcal disease in infants are also seen (9), to determine if these isolates are genetically related. For comparison, 6B isolates from Georgia, Maryland, Massachusetts, Oklahoma, Ohio, Texas, Washington, and Wisconsin were included.

MATERIALS AND METHODS

Bacterial isolates. Sixteen Alaskan isolates of serotype 6B were selected from pneumococci submitted to the Arctic Investigations Program, located in Anchorage, Alaska, as part of statewide surveillance. This group of isolates included penicillin-susceptible and intermediate isolates, represented a range of susceptibilities to three other antibiotics (TMP-SMZ, erythromycin, and tetracycline), were from patients from various service units of the state (Anchorage, 2; Interior, 1; YKD, 13), and were spread over a period of 10 years (1982 to 1992). Fourteen pneumococcal isolates were from Native Americans from the White Mountain Apache Reservation in Arizona (Mathirum Santosham, Johns Hopkins University Center for American Indian and Alaska Native Health). Eleven isolates obtained from Washington (Douglas Black, Surveillance Group for Drug Resistant Streptococcus pneumoniae in Washington State) and 18 isolates (Georgia, 4; Maryland, 1; Massachusetts, 1; Oklahoma, 2; Ohio, 3; Texas, 6; Wisconsin, 1) submitted to the Division of Bacterial and Mycotic Diseases, Centers for
Disease Control and Prevention, Atlanta, Georgia, were also analyzed. All isolates were serotype 6B, and most were from children less than 3 years old.

**Antimicrobial susceptibility testing.**Susceptibility testing of 6B isolates from Alaska, Arizona, Georgia, Maryland, Massachusetts, Oklahoma, Ohio, Texas, and Wisconsin was performed by the standard agar dilution method, as described by the National Committee for Clinical Laboratory Standards (NCCLS) (25). The following antibiotics were tested: penicillin, erythromycin, TMP-SMZ, and tetracycline. The susceptibilities of isolates from Washington were determined by broth microdilution methods, as described by NCCLS (25), with the exception of tetracycline, the susceptibilities for which were determined by the ability of the isolates to grow in the presence of 10 μg of the antibiotic per ml. The MIC was determined to be the lowest concentration of antibiotic that inhibited growth.

For the purposes of this study, penicillin-intermediate isolates were defined as having a MIC of 0.125–2 μg/ml, penicillin-resistant isolates were defined as having a MIC of ≥2 μg/ml. TMP-SMZ-intermediate and -resistant isolates were defined as having MICs between 1/19 and 2/38 μg/ml and ≥4/76 μg/ml, respectively. Resistance to tetracycline was defined as a MIC of ≥1 μg/ml; erythromycin-intermediate and -resistant isolates were defined as having MICs of 1 to 2 μg/ml and ≥4 μg/ml, respectively, with NCCLS breakpoints (23).

**DNA preparation and restriction enzyme digestion.** Bacteria were grown for 18 to 22 h at 37°C in 5% CO₂ on Brucella agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep blood. Bacterial cells were suspended in 2 ml of Mueller-Hinton broth (Difco) to an optical density at 560 nm adjusted to read between 0.4 and 0.6. The cells were centrifuged at 10,000 rpm for 2 min at room temperature and washed with 1.0 ml of buffer A [10 mM Tris, 1 mM NaCl (pH 8.0)]. The bacterial pellets were resuspended in 500 μl of low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) prepared in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8.0]) held at 50°C. Three hundred microliters of each bacterial suspension was poured into Plexiglas molds (Bio-Rad). After solidification, bacteria embedded in the agarose blocks were incubated in 5 ml of TE buffer [0.01 M Tris, 0.001 M EDTA (pH 8.0)] for 18 to 22 h at 37°C in 5% CO₂ on Brucella agar plates (Difco Laboratories, Detroit, Mich.).

**PFGE.** The digested DNA plugs were placed in wells of a 1% agarose gel (SeaKem; FMC Corp., Rockland, Maine) prepared in 0.5× TBE (pH 8.0) and sealed with 1% low-melting-point agarose at 50°C. The digested DNA plugs were electrophoresed in a contour-clamped homogeneous electric field apparatus (CHEF DRII; Bio-Rad) with initial to final switch times ranging from 1 to 15 s.

**Analysis of pneumococcal DNA by PFGE.** The stability of *Apal* and *Sma*I PFGE patterns was determined by selecting one isolate (4463) and looking at the PFGE patterns before and after 50 in vitro passages (data not shown). In addition, reproducibility of the PFGE patterns was established by repeated testing of the same isolate on separate occasions on different gels; all such tests yielded identical PFGE patterns, suggesting stability of the PFGE patterns. All isolates were digested with *Apal* and *Sma*I, and the PFGE banding patterns were visually compared. Figures 1A and B show representative PFGE pat-
terns of DNA digested with *Apa*I and *Sma*I, respectively, from isolates taken from the different geographic locations. Thirteen of the 16 Alaskan isolates showed indistinguishable PFGE patterns (A1 S1), while one isolate (4457) was highly related (CS = 97%), showing one extra band after restriction with *Apa*I (A1), and an identical *Sma*I pattern (S1) (Table 2). Among the 14 isolates from Arizona, 1 isolate (1130) was indistinguishable from the predominant *Apa*I and *Sma*I PFGE patterns seen in the Alaskan isolates (A1 S1); 5 others (2398, 2402, 2404, 2396, and 2399) were highly related (A1 plus one band between 145 and 242 kb, A2 minus one band between 48.5 and 97 kb, S12 minus one band between 97 and 145.5 kb, and S13 plus one band between 48.5 and 97 kb) (CS, 90 to 97%). Thus, these 20 isolates from Alaska and Arizona are likely to have had a common ancestor, and we believe they represent a clone.

Six additional combined PFGE profiles were seen in the remaining eight isolates from Arizona (Table 2). Of these, isolates 1133 (A4 S6) and 1134 (A4, S6) were indistinguishable from each other except for an extra band in 1133 after cutting with *Apa*I. In addition, isolates 2400 (A6 S3) and 1131 (A6 S3) were indistinguishable.

Among the 28 isolates from the other eight states, 22 additional combined PFGE patterns were present. Of these, several clusters of isolates with related PFGE patterns were identified. The first cluster included isolates 3371 (A10 S14) and 2772 (A10 S14) from Texas, which were indistinguishable except for one extra band after restriction with *Sma*I (CS = 95%), and two isolates (2946 [A10, S13]) and 25 [A10, S5]) from Washington. These four isolates had highly related *Apa*I PFGE patterns (A10, A10, A10) but different *Sma*I PFGE patterns (CS = 83%). The two isolates from Washington appeared to be quite different after restriction with *Sma*I (CS = 78%) and therefore are less likely to be related to each other.

Each *Apa*I and *Sma*I PFGE pattern was assigned a letter and number designation (A1 to A26, S1 to S20); isolates having patterns that differed from these patterns by one to three bands were considered closely related and were clustered into subtypes, e.g., A4.
or the other two isolates in this cluster. Another cluster of isolates (2773, 1456, 467, and 3205) had highly related \textit{ApaI} (\textit{A11, A11}), PFGE patterns. Three of these isolates were from Georgia and were indistinguishable after restriction with \textit{SmaI} (\textit{S8}), while isolate 2773 from Texas showed a similar \textit{SmaI} PFGE pattern (\textit{S8}). The third cluster included isolates 1153, 1247, 171, 174, and 2494, with highly related \textit{ApaI} (\textit{A19, A19}), (CS = 96\%) PFGE patterns but different \textit{SmaI} PFGE patterns (\textit{S1, S8}, \textit{S12, S12}), (CS, 80 to 83\%).

Correlation between antibiogram and PFGE patterns. Of the cluster of 20 isolates with related PFGE patterns, 13 (65\%) were intermediate to penicillin; the remaining 7 were susceptible. Eleven of these isolates were intermediate or fully resistant to three or more of the antibiotics tested (profiles \textit{C, E, G}). The five isolates that shared the PFGE \textit{ApaI} pattern \textit{A1}, were susceptible to all of the antibiotics tested, except for one isolate (4457) which was intermediate to penicillin (Table 2). All five isolates were from Arizona patients.

Among the 16 isolates from Alaska, three predominant PFGE patterns (\textit{A1 S1, A2 S2, and A3 S1}), and nine different antibiograms were seen (Tables 1 and 2). Fourteen (88\%) of these isolates were intermediate to penicillin and showed seven different antibiotic susceptibility profiles to the other three antibiotics tested. The remaining two isolates, 917 and 4463, were susceptible to penicillin but resistant to one other antibiotic (profiles \textit{D} and \textit{H}).

Among the 14 isolates from Arizona, seven PFGE patterns (\textit{A1 S1, A4 S6, A5 S5, A6 S3, A7 S4, A8 S5, and A9 S7}) and four different antibiograms (profiles \textit{A, B, D}, and \textit{F}) were seen. Seven (50\%) of these isolates were fully susceptible to all four antibiotics tested. Five (36\%) were intermediate to penicillin and fully susceptible to the other three antibiotics.

The remaining 28 isolates had 22 combined \textit{ApaI} plus \textit{SmaI} PFGE patterns and were found in all 10 of the different antibiograms (Tables 1 and 2). Sixteen (57\%) of these isolates were susceptible to penicillin; the remaining 12 were either intermediate or resistant to penicillin. Within the first PFGE cluster (3371, 2272, 2946, and 25), all isolates were penicillin nonsusceptible resistant to TMP-SMZ, and susceptible to tetracycline. Within the second PFGE cluster (2773, 1456, 467, and 3205), all isolates were resistant to penicillin, TMP-SMZ, and erythromycin. Antibiograms within the third PFGE cluster (1153, 1247, 171, 174, and 2494) were variable, ranging from fully susceptible to multidrug resistant (Table 2).

**DISCUSSION**

Phenotyping and genotyping methods are increasingly being used to monitor the source and transmission of disease, as well as the emergence of strains with increased pathogenicity. While serotyping and antibiotic susceptibility testing have been the most common epidemiologic tools for typing pneumococci, they have relatively limited discriminatory power (2). In contrast, genotyping methods such as multilocus enzyme electrophoresis (MLEE) (8, 15, 22, 27, 32), ribotyping (19, 29), and PFGE (5, 12, 18) have shown greater discriminatory power for isolates within a single serogroup and have provided evidence for clonality and intercontinental spread of particular drug-resistant pneumococci (8, 11, 19, 21, 25, 26). In this study, we utilized PFGE following \textit{ApaI} and \textit{SmaI} restriction digestion of chromosomal DNA to determine genetic relatedness between pneumococcal serotype 6B isolates from different geographic locations in the United States.

The combined PFGE profiles observed in the Alaskan isolates indicated that these 16 isolates are ancestrally related (CS, 90 to 97\%), suggesting a common origin. These isolates with the combined \textit{ApaI} and \textit{SmaI} pattern A1 S1 were recovered over a 10-year period and had variable antibiograms, with the majority (88\%) showing intermediate resistance to penicillin. This suggests that combined PFGE patterns can be relatively stable over a number of years, even as antibiograms change. Six of the 14 isolates from Native American patients from Arizona had \textit{ApaI} and \textit{SmaI} PFGE patterns that were highly related to those of the Alaskan isolates (CS, 90 to 97\%), suggesting a common ancestral origin. However, these six isolates, with the exception of one (1130), were fully susceptible to all of the antibiotics tested, suggesting that one penicillin-susceptible Alaskan isolate (917) may represent the ancestral organism for these penicillin-susceptible Arizona isolates.

The PFGE patterns of the remaining isolates were heterogeneous, with no more than five isolates having the same \textit{ApaI} or \textit{SmaI} pattern (CS = 93\%) and no more than four isolates having the same combined \textit{ApaI} and \textit{SmaI} PFGE patterns (CS = 93\%) (Table 2). This is comparable with other studies that have shown that isolates of the same serotype are not necessarily more closely related to each other than isolates of different serotypes (5, 8, 11, 16, 26, 27).

To increase the discriminatory power of PFGE, we used two enzymes for typing purposes. In several cases (the smaller clusters of four to five isolates), the use of three enzymes would have provided greater discriminatory power, as previously described for other bacterial species (33). \textit{SmaI} digestion, which has been used by a number of laboratories (3, 5, 16, 19, 27, 30, 34), was found to be less discriminatory than \textit{ApaI} digestion for these isolates.

MLEE has been previously used to subtype pneumococcal isolates (12, 19, 21, 27, 32). Versalovic et al. (32), using MLEE, reported that penicillin-resistant serotype 6B isolates from Alaska were genetically related to Spain, Iceland, and Texas 6B clones. These researchers suggested that common resistant isolates from diverse locations share a recent ancestor or that isolates of a particular phylogenetic lineage are predisposed to develop penicillin resistance. In contrast, penicillin-susceptible pneumococcal 6B isolates presented a heterogeneous collection of multilocus enzyme genotypes (21, 32). While MLEE analysis detects mutations in a variety of genes for metabolic enzymes throughout the entire chromosome, the polymorphism obtained with PFGE fingerprints has been found to be greater than that obtained with MLEE analysis (17); thus, PFGE has the potential to further subdivide pneumococcal 6B clones. These researchers suggested that common resistant isolates of a particular phylogenetic lineage are predisposed to develop penicillin resistance. In contrast, penicillin-susceptible pneumococcal 6B isolates presented a heterogeneous collection of multilocus enzyme genotypes (21, 32). While MLEE analysis detects mutations in a variety of genes for metabolic enzymes throughout the entire chromosome, the polymorphism obtained with PFGE fingerprints has been found to be greater than that obtained with MLEE analysis (17); thus, PFGE has the potential to further subdivide pneumococcal 6B clones. Isolates of a particular phylogenetic lineage are predisposed to develop penicillin resistance. In contrast, penicillin-susceptible pneumococcal 6B isolates presented a heterogeneous collection of multilocus enzyme genotypes (21, 32).

**ACKNOWLEDGMENTS**

We thank S. Deliganis, from Northwest Pharmaceutical Research Network, and T. Fritische, from Laboratory Medicine, University of Washington, Seattle, for providing isolates.

**REFERENCES**


