A Novel Multiresistant Streptococcus pneumoniae Serogroup 19 Clone from Washington State Identified by Pulsed-Field Gel Electrophoresis and Restriction Fragment Length Patterns

VICKI A. LUNA, DANIEL B. JERNIGAN, ALAN TICE, JAMES D. KELLNER, and MARIYLN C. ROBERTS*
Department of Pathobiology, University of Washington, Seattle, and Infections Limited, Tacoma; National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; and Departments of Pediatrics and Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada

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In 1997, a cluster of multiresistant invasive serogroup 19 pneumococcus infections, including two fatalities, was reported in Washington State. Further investigation identified other cases. Fourteen Washington Streptococcus pneumoniae isolates, four from Alaska, and eight isolates from eastern Canada with reduced penicillin susceptibility (MIC of ≥1 μg/ml) were included in the study. Pulsed-field gel electrophoresis (PFGE) with Apal, SacII, and Smal restriction enzymes and IS1167 and mef restriction fragment length polymorphism (RFLP) pattern analysis were performed. Twenty of the 26 isolates had identical or related PFGE patterns, with two or all three enzymes, and identical or related IS1167 RFLP patterns, indicating that they were genetically related. These 20 isolates contained the mef gene conferring erythromycin resistance and had identical mef RFLP patterns. The PFGE and RFLP patterns were distinct from those of multiresistant clones previously described and suggest that a new multiresistant clone has appeared in Washington, Alaska, and eastern Canada. This newly characterized clone should be included in the Pneumococcal Molecular Epidemiology Network.

Streptococcus pneumoniae is the leading bacterial cause of community-acquired pneumonia, otitis media, bacteremia, and meningitis in the United States (14). In the past 20 years, a worldwide increase in the incidence of antibiotic-resistant S. pneumoniae has been observed (3, 13, 30). Although more than 90 serotypes of S. pneumoniae exist, resistance to two or more different classes of antibiotics (i.e., multiresistance) is currently limited to a few major serotypes (6B, 9V, 14, 19F, and 23F) (11, 12, 30). The first non-penicillin-susceptible multiresistant S. pneumoniae strain, described in the 1970s, contained a conjugative transposon, Tn1545, which carried four resistance genes: erm (B) (macrolides, lincosamides, and streptogramin B), tet (M) (tetracycline), aph (A)-3 (aminoglycosides), and cat (chloramphenicol). This family of transposons has since disseminated through the pneumococcal population (5, 8). Since the first description of an S. pneumoniae clone, Spain-23F-1, other clones have been identified (18, 30). A Pneumococcal Molecular Epidemiology Network has been newly established to collect, study, and assign systematic number designations to S. pneumoniae clones that meet the Network’s criteria (K. Klugman, Letter, ASM News 64:371, 1998).

In February 1997, the Washington State Health Department was notified of three cases of pneumonia due to ceftriaxone-resistant S. pneumoniae, two of which were fatal. Further investigation found that all three invasive isolates were from patients in the same community. The isolates were serogroup 19, were nonsusceptible to penicillin, and were resistant to ceftriaxone, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole. Although serogroup 19 represents approximately 10% of pneumococci tested in Washington, ceftriaxone-resistant S. pneumoniae had only rarely been identified in Washington (2, 10; Centers for Disease Control and Prevention [CDC], unpublished data). To determine the magnitude of the problem and to further characterize the isolates, we selected 14 serogroup 19 multiresistant S. pneumoniae isolates collected from the hospitals in the community where the original cluster occurred and from other hospitals throughout Washington State. We also included four randomly chosen Alaskan isolates from a pool of multiresistant serotype 19F isolates. We also chose eight Canadian isolates, serogroup 19, which had antibiograms, including resistance to erythromycin, cephalosporins, and penicillin, similar to those of the Washington isolates for comparison. These 26 isolates were compared with previously characterized multiresistant S. pneumoniae clones using pulsed-field gel electrophoresis (PFGE) and insertion sequence (IS) restriction fragment length polymorphism (RFLP) pattern typing.

(This study was presented in part as abstract 10638 at the Eighth International Congress of Infectious Diseases in Boston, Mass., May 1998, where it won the North American Pasteur-Merieux Connought Award in Epidemiology.)

MATERIALS AND METHODS

Bacteria. We examined 26 S. pneumoniae serogroup 19 isolates with diminished susceptibility to penicillin (MIC of >1 μg/ml) and resistance to at least three other antibiotics (Table 1). Seven isolates, including the initial outbreak cluster, were from three hospitals around Tacoma, Wash., and were collected during February and March 1997 (WA1 to WA7). Seven isolates were from other hospitals in Washington and were collected between December 1995 and April 1996 in a prior survey (WA8 to WA14). The Washington isolates were from adults; most were from hospitals in the Puget Sound region, which includes Tacoma and represents the major portion of the state’s population. The Arctic Investigations Program (AIP), National Center for Infectious Disease, CDC, provided four serogroup 19 isolates from adults treated at hospitals in Alaska (AK15 to AK18). The Alaskan isolates were randomly chosen from a pool of multiresistant serotype 19F isolates. Eight isolates (CN19 to CN26) from children in metropolitan Toronto and the neighboring urban Peel region of Canada were chosen because they were serogroup 19 and had antibiograms similar to...
those of the Washington isolates, being resistant to erythromycin, azithromycin, and clindamycin before and after exposure to a low level of erythromycin (0.5 μg/ml) to identify indubitably resistant isolates (17). Two S. pneumoniae strains, ATCC 6305 and ATCC 40619, were used as controls. The MIC breakpoints were available from the National Committee for Clinical Laboratory Standards for all of the antibiotics except loracarbef, grepafloxacin, linezolid, and HMR3647 (19).

**Media and growth conditions.** Bacteria were grown on brucella blood agar (Difco) supplemented with 5% sheep red blood cells and incubated for 18 to 24 h with 5% CO₂ at 36.5°C. Mueller-Hinton agar (Difco) supplemented with 5% sheep red blood cells and appropriate concentrations of antibiotics was used for the agar dilutions (19). Bacterial stocks were maintained at −70°C in sterile skim milk. Aliquots were subcultured onto appropriate media from the frozen stocks as needed. Purity was maintained by stringent aseptic techniques and confirmed by biochemical methods as described previously (25).

**PFGE analysis.** Bacteria were grown for 18 to 24 h on brucella blood agar plates at 36.5°C in CO₂, harvested, and made into blocks with 1% low-melting-point agarose (Bio-Rad Laboratories, Richmond, Calif.) in a PFGE mold provided by the manufacturer (Bio-Rad) as previously described (16, 23). The blocks were digested with proteinase K (Sigma) (100 μg/ml), washed, and stored at 4°C as described previously (16, 23). Gel plugs (approximately 3 by 6 mm) were cut from the blocks and digested for 20 to 24 h with 35 U of Apal (Promega, Madison, Wis.) or SacII (Promega) at 37°C or Smal (Promega) at 25°C as described previously (16, 23).

The digested gel blocks were embedded in a 1% agarose gel (SeaKem; FMC Corporation, Rockland, Maine) prepared with 1× TBE (Tris-buffered saline) (pH 8.0) and run using a CHEF-DRII (contour-clamped horizontal electrophoresis) apparatus (Bio-Rad) at 175 V for 20 h for Smal-digested DNA gel plugs and 22 h for SacII- and Apal-digested gel plugs. DNA bands were visualized by ethidium bromide and UV light and photographed as previously described (23, 31).

Analysis of the PFGE patterns was performed by visual inspection of the photographs. Apal and SacII produced PFGE patterns of 10 to 15 DNA bands between 45.5 and 291.5 kb, while Smal digests produced PFGE patterns with 8 to 12 DNA bands between 45.5 and 291.5 kb. The most common PFGE patterns were designated with the first letter of the enzyme and an assigned number (A37 to A50 for Apal and S26 to S37 for Smal). We did not start with A1 because those PFGE patterns have previously been assigned to S. pneumoniae isolates from other parts of the United States (23). To distinguish SacII from Smal patterns, C was used for the designated name for SacII patterns (C8 to C21). Isolates with DNA patterns that differed by three or fewer bands from the main pattern were considered to be related and given a subscribed number starting with 1 (A37, A38, etc.), as the band difference could be explained by one genetic event (23, 31). Isolates which had a difference of more than three DNA bands were considered unrelated and were given consecutive numbers as they appeared (A38, A39, A40, etc.). Isolates that were identical or highly related (three or fewer bands) by two or three restriction enzyme PFGE patterns were considered to be genetically related. We have found this criterion valuable in other studies with S. pneumoniae as well as for other pathogens (23, 31). This classification is more stringent than the five-band difference previously suggested by Tenover et al. (29).

**RESULTS**

**Antibiogram analysis.** Non-susceptibility to penicillin was a criterion for inclusion in the study. The 22 Washington and Canadian isolates were also generally resistant to the cephalosporins. Cefprozil, cefazidime, and loracarbef showed the
TABLE 2. Antibiogram of Washington and Canadian *S. pneumoniae* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (µg/ml) of drug&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>W45</td>
<td>Pen 0.03, Ceftri 0.03, Ctax 0.03, Cefpro 0.03, Cefotax 0.03, Lora 0.03, Eryth 0.03, Azith 0.03, Clin 0.03, Cipro 0.03, Grepa 0.03, Levo 0.03, Spar 0.03, Trova 0.03, Linez 0.03, HMR 0.03</td>
</tr>
<tr>
<td>W46</td>
<td>Pen 0.03, Ceftri 0.03, Ctax 0.03, Cefpro 0.03, Cefotax 0.03, Lora 0.03, Eryth 0.03, Azith 0.03, Clin 0.03, Cipro 0.03, Grepa 0.03, Levo 0.03, Spar 0.03, Trova 0.03, Linez 0.03, HMR 0.03</td>
</tr>
<tr>
<td>W47</td>
<td>Pen 0.03, Ceftri 0.03, Ctax 0.03, Cefpro 0.03, Cefotax 0.03, Lora 0.03, Eryth 0.03, Azith 0.03, Clin 0.03, Cipro 0.03, Grepa 0.03, Levo 0.03, Spar 0.03, Trova 0.03, Linez 0.03, HMR 0.03</td>
</tr>
<tr>
<td>W48</td>
<td>Pen 0.03, Ceftri 0.03, Ctax 0.03, Cefpro 0.03, Cefotax 0.03, Lora 0.03, Eryth 0.03, Azith 0.03, Clin 0.03, Cipro 0.03, Grepa 0.03, Levo 0.03, Spar 0.03, Trova 0.03, Linez 0.03, HMR 0.03</td>
</tr>
<tr>
<td>W49</td>
<td>Pen 0.03, Ceftri 0.03, Ctax 0.03, Cefpro 0.03, Cefotax 0.03, Lora 0.03, Eryth 0.03, Azith 0.03, Clin 0.03, Cipro 0.03, Grepa 0.03, Levo 0.03, Spar 0.03, Trova 0.03, Linez 0.03, HMR 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pen, penicillin; Ceftri, ceftriaxone; Ctax, cefotaxime; Cefpro, cefprozil; Cefotax, cefotaxime; Lora, loracarbef; Eryth, erythromycin; Azith, azithromycin; Clin, clindamycin; Cipro, ciprofloxacin; Grepa, grepafloxacin; Levo, levofloxacin; Spar, sparfloxacin; Trova, trovafloxacin; Linez, linezolid; HMR, HMR3647. Breakpoints (in micrograms per milliliter) per National Committee for Clinical Laboratory Standards guidelines (19), unless otherwise noted, are as follows. Penicillin: susceptible, ≤0.06; intermediate, 0.1; resistant, ≥2; ceftriaxone: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; cefotaxime: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; cefprozil: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; cefotaxime: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; clindamycin: susceptible, ≤0.25; intermediate, 0.5; resistant, ≥2; ciprofloxacin: susceptible, ≤0.25; intermediate, 0.5; resistant, ≥2; sparfloxacin: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; trovafloxacin: susceptible, ≤0.25; intermediate, 0.5; resistant, ≥2; linezolid: ≤8; intermediate, 16; resistant, ≥32. The breakpoints for cefotaxime, ceftriaxone, clindamycin, and trovafloxacin are based upon the breakpoints of similar antibiotics (ceftriaxone and levofloxacin). Antibiograms of the four Alaskan isolates (AK14 to AK18) were determined by alternate methods; however, the isolates were resistant to penicillin, the cephalosporins, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (data not shown) and carried the *erm* genes.

<sup>b</sup> Isolate WA9 contained both the *erm* and *ermB* genes. MICs were determined after the isolate was exposed to a low level of erythromycin (0.5 µg/ml), with corresponding MICs of erythromycin, azithromycin, and clindamycin being 128 µg/ml (17).

The six remaining *S. pneumoniae* isolates, one Washington isolate (WA6) and five Canadian isolates (CN21, CN23, CN24, CN25, and CN26), had distinct PFGE patterns with each of the three enzymes and were not considered to be genetically related to the 20 isolates described above (Table 3).

**PFGE comparison of related isolates with multiresistant clones.** Isolate WA8, with the most common PFGE pattern for all three enzymes (A37, C8, and S26), was selected as a representative of the related isolates and was compared to the six previously known, multiresistant clones (Fig. 1). Five of the six clone isolates had unique PFGE patterns with all three enzymes from the PFGE patterns of isolate WA8. Isolate SP27 (267-Spain-23F-1) had distinct PFGE patterns with ApaI and SacII enzymes (Fig. 1) and a three-band difference for the Smal PFGE pattern compared to the *Smal* PFGE pattern of isolate WA8.

**IS1167 RFLP analysis.** Of the 20 isolates with the same or related PFGE patterns, restriction fragment length patterns...
were either identical or had a one-band difference in the IS1167 RFLP patterns. The six clone isolates (SP27 to SP32) carried the \textit{mef} gene, and RFLP analysis could not be performed.

### DISCUSSION

PFGE analysis using three different enzymes and IS1167 \textit{mef} RFLP typing identified a unique, multiresistant, serogroup 19 \textit{S. pneumoniae} group of 20 isolates. This clone was characterized by reduced susceptibility to penicillin and resistance to extended-spectrum cephalosporins, erythromycin, and ciprofloxacin. Recent reports have indicated increases in ceftriaxone-resistant pneumococcus-caused illness in Washington (D. B. Jernigan, I. Kargacin, A. Poole, and J. Kobayashi, Program Abstr. 36th Annu. Meet. Infect. Dis. Soc. Am., abstr. 565-Sa, 1998). This newly identified clone most likely contributed to these increases. The Washington isolates were genetically related to the multiresistant isolates obtained from Alaska and eastern Canada but unrelated to five previously characterized, multiresistant clones (30). The Spanish isolate (SP27) representing the Spanish clone (Spain-23F-1) differed from the Washington clone in PFGE patterns with two enzymes, \textit{ApaI} (Fig. 1) and \textit{SacII}, and in IS1167 RFLP typing, indicating that it was completely different from the Washington clone. In previous work with \textit{S. pneumoniae} and \textit{Neisseria gonorrhoeae}, if any two isolates had an identical or related PFGE pattern with only one of three enzymes used, we did not consider the isolates to be related (23, 31).

The initial three isolates that prompted our investigation were fully resistant to penicillin, cefotaxime, erythromycin, and other antibiotics but were susceptible to vancomycin, the newer quinolones (grepafloxacin, levofloxacin, and trovafloxacin), linezolid, and the investigational antibiotic HMR3647 (Table 2). Reports of these multiresistant isolates in Tacoma, with their associated mortality, presented a challenge to clinicians choosing empiric treatment for severe community-acquired pneumonia. In regions where multiresistant isolates have been identified, clinicians may choose to request that vancomycin, newer quinolones, or linezolid be added to routine laboratory susceptibility testing of invasive \textit{S. pneumoniae} isolates. In addition, the new compound HMR3647 may offer another therapeutic choice in the future.
A recent report from Spain has described a serogroup 19 isolate that is nonsusceptible to penicillin and resistant to ceftaxime (24). Comparison of the Washington clone to this isolate would be of interest. Surveillance of invasive pneumococcal illness in Washington is continuing, which will allow us to monitor trends in antibiotic resistance and to detect any further increase in multiresistant pneumococcal infections. Based on our results, we suggest that this newly characterized clone has all of the characteristics required to be included in the recently organized Pneumococcal Molecular Epidemiology Network as Washington 19-14 (K. Klugman, Letter).

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