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

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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 *Apa*I, *Sac*II, and *Sma*I restriction enzymes and IS*1167* 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 IS*1167* 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 six 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, Tn*1545*, which carried four resistance genes: *erm*(B) (macrolides, lincosamides, and streptogramin B), *tet*(M) (tetracycline), *aphA*-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 Connaught 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

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TABLE 1. Characteristics of *S. pneumoniae* isolates

Isolate[a]	Date isolated (mo/yr)[b]	Specimen source	Serotype
Related isolates			
WA1	2/97	Blood	19F
WA2	2/97	Blood	19F
WA3	2/97	Blood	19F
WA4	3/97	Nasopharynx	19F
WA5	3/97	Sinus	19F
WA7	3/97	Sputum	19
WA8	12/95	Sputum	19F
WA9	12/95	Sputum	19A
WA10	12/95	Blood	19F
WA11	1/96	Sinus	19F
WA12	4/96	Wound	19F
WA13	6/96	Blood	19F
WA14	6/96	Unknown	19F
AK15	4/94	Nasopharynx	19F
AK16	8/94	Blood	19F
AK17	2/96	Blood	19F
AK18	?/97	Blood	19F
CN19	4/95	Nasopharynx	19F
CN20	4/95	Nasopharynx	19F
CN24	1/96	Nasopharynx	19F
Unrelated isolates			
WA6	2/97	Sputum	19
CN21	5/95	Nasopharynx	19F
CN22	6/95	Nasopharynx	19F
CN23	11/95	Nasopharynx	19F
CN25	1/95	Blood	19F
CN26	4/95	Blood	19F
Multiresistant clones			
SP27 (267-Spain-23F-1)			23F
SP28 (681-Spain-6B-2)			6B
SP29 (665-France-9V-3)			9V
SP30 (17219-South Africa-19A-7)			19A
SP31 (50803-South Africa-6B-8)			6B
SP32 (51702-South Africa-19A-unnumbered)			19A

[a] AK, Alaska; CN, Canada; WA, Washington State.
[b] ?, month unknown.

those of the Washington isolates, being resistant to erythromycin, cephalosporins, and penicillin. Some clinical and antimicrobial susceptibility data from the Canadian isolates have been previously reported (15). The isolates outside of Washington allowed us to determine if genetically related *S. pneumoniae* strains were present in regions outside Washington State. In addition, six isolates representing six known, characterized clones (267-Spain-23F-1, 681-Spain-6B-2, 665-France-9V-3, 17219-South Africa-19A-7, 50803-South Africa-6B-8, and 51702-South Africa-19A-unnumbered) were provided by the Pneumococcal Diseases Research Unit at the South African Institute for Medical Research, University of the Witwatersrand, Johannesburg, South Africa (SP27 to SP32).

Serology. Serogroups were determined by the Quellung reaction (25) by our laboratory and confirmed by the University of Washington Medical Center and/or the AIP. Subtyping was performed by counterimmunodiffusion electrophoresis at the AIP.

Antibiograms. The MIC was determined using agar dilution following the National Committee for Clinical Laboratory Standards guidelines (9, 19). The Washington and Canadian isolates were tested with the following antibiotics: penicillin, cefotaxime, ceftriaxone, cefprozil, ceftazidime, loracarbef, erythromycin, azithromycin, clindamycin, ciprofloxacin, grepafloxacin, levofloxacin, sparfloxacin, trovafloxacin, linezolid, and HMR3647 (a new ketolide) (Table 2). Isolates from Alaska were not included in the full antibiogram determination.

Ceftriaxone was obtained from Difco (Detroit, Mich.). Clindamycin, erythromycin, cefotaxime, and penicillin were purchased from Sigma Chemical Co. (St. Louis, Mo.). The other antibiotics were provided by manufacturers as follows: ciprofloxacin, Bayer Corp., West Haven, Conn.; cefprozil, Bristol-Myers Squibb, Princeton, N.J.; grepafloxacin and ceftazidime, Glaxo-Wellcome Co., Triangle Park, N.C.; levofloxacin and HMR3647, Hoechst Marion Roussel, Paris, France; trovafloxacin and azithromycin, Pfizer, Inc., Groton, Conn.; loracarbef, Eli Lilly and Company, Indianapolis, Ind.; and linezolid, Pharmacia Upjohn, Kalamazoo, Mich. The antibiotic concentrations tested ranged from 0.002 to 16 μg/ml for the quinolones and ketolides and from 0.031 to 128 μg/ml for the other antibiotics. The bacterial isolates were tested against erythromycin, azithromycin, and clindamycin before and after exposure to a low level of erythromycin (0.5 μg/ml) to identify inducibly 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% CO_2 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 CO_2, 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 *Apa*I (Promega, Madison, Wis.) or *Sac*II (Promega) at 37°C or *Sma*I (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 0.5× Tris-borate-EDTA (TBE) (pH 8.0) and run using a CHEF-DRII (contour-clamped horizontal electrophoresis) apparatus (Bio-Rad) at 175 V for 20 h for *Sma*I-digested DNA gel plugs and 22 h for *Sac*II- and *Apa*I-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. *Apa*I and *Sac*II produced PFGE patterns of 10 to 15 DNA bands between 45.5 and 291.5 kb, while *Sma*I 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 *Apa*I and S26 to S37 for *Sma*I). We did not start with A1 because these PFGE patterns have previously been assigned to *S. pneumoniae* 6B isolates from other parts of the United States (23). To distinguish *Sac*II from *Sma*I patterns, C was used for the designated name for *Sac*II 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 subscripted number starting with 1 ($A37_1$, $A38_2$, 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).

IS*1167* RFLP typing. Whole-cell DNA extracts were prepared from isolates grown in 100 ml of brain heart infusion broth supplemented with 0.6% D-glucose plus 0.03% DL-threonine and incubated at 36.5°C in 5% CO_2, as previously described (1, 16, 17). DNA was digested with 80 U of *Hin*dIII restriction enzyme and run on a 0.7% agarose gel in 0.5× TBE buffer at 100 V for 3.0 h (26). Southern blots were prepared from the agarose gels and hybridized with a ^{32}P-labeled oligonucleotide probe, DAMO13 (5′-TGG ATA TTA TGG AGC CT-3′) (22, 32). This probe is specific for an upstream region of insertion sequence IS*1167* (22, 32). The bands were counted, and patterns that differed in band number or size of band by more than one band were considered unrelated. RFLP typing and analysis were performed multiple times.

***mef* RFLP typing.** Whole-cell DNA extracts were prepared from isolates grown in 100 ml of brain heart infusion as described for the IS*1167* RFLP typing. DNA was digested with 80 U of *Hin*dIII restriction enzyme and run on a 0.7% agarose gel in 0.5× TBE buffer at 100 V for 3.0 h (26). Southern blots were prepared from the agarose gels and hybridized with a ^{32}P-labeled oligonucleotide probe, MF5 (5′-GGT GCT GTG ATT GCA TCT ATT AC-3′), that is specific for the *mef* gene (17, 28). The bands were counted and analyzed as described for the IS*1167* RFLP analysis. RFLP typing and analysis were performed multiple times. This is the first time that the *mef* gene has been used for RFLP typing.

RESULTS

Antibiogram analysis. Nonsusceptibility to penicillin was a criterion for inclusion in the study. The 22 Washington and Canadian isolates were also generally resistant to the cephalosporins. Cefprozil, ceftazidime, and loracarbef showed the

TABLE 2. Antibiogram of Washington and Canadian *S. pneumoniae* isolates

Isolate	MIC (μg/ml) of drug[a]:															
	Pen	Ceftri	Ctax	Cefpro	Ceftaz	Lora	Eryth	Azith	Clin	Cipro	Grepa	Levo	Spar	Trova	Linez	HMR
Related isolates																
WA1	2	2	2	16	16	128	4	2	0.03	16	0.25	1	0.5	0.125	1	0.002
WA2	2	1	2	16	16	64	4	4	0.016	16	0.25	1	0.5	0.06	1	0.002
WA3	2	2	2	16	16	128	8	2	0.03	16	0.25	1	0.5	0.06	1	0.016
WA4	2	1	2	16	16	128	4	2	0.03	16	0.25	1	0.5	0.125	1	0.002
WA5	2	1	2	16	16	64	4	2	0.03	16	0.25	1	0.25	0.125	1	0.002
WA7	2	1	2	16	16	64	4	4	0.125	16	0.25	1	1	0.25	1	0.004
WA8	1	1	1	16	32	64	4	2	0.03	8	0.25	1	0.5	0.125	1	0.002
WA9	1	1	1	16	16	64	2[b]	4[b]	0.06[b]	8	0.25	1	1	0.125	1	0.002
WA10	2	1	1	16	16	64	4	4	0.03	8	0.125	1	0.5	0.25	0.25	0.016
WA11	1	1	1	16	16	128	4	4	0.03	16	0.008	1	0.5	0.25	1	0.002
WA12	1	1	1	16	16	64	2	2	0.03	8	0.125	1	0.5	0.25	1	0.016
WA13	1	1	1	8	16	64	2	2	0.06	16	0.125	0.5	0.5	0.25	1	0.016
WA14	1	0.5	1	16	16	64	4	2	0.03	8	0.125	1	0.5	0.25	1	0.002
CN19	2	1	1	16	32	128	2	2	0.25	1	0.25	1	0.25	0.125	1	0.016
CN20	2	1	1	8	32	64	1	1	0.25	0.5	0.25	0.5	0.125	0.125	1	0.016
CN24	2	0.5	0.03	8	32	64	1	2	0.25	0.5	0.25	1	0.25	0.125	1	0.032
Unrelated isolates																
WA6	1	1	1	0.25	0.25	0.5	1	0.25	0.016	0.125	0.062	0.5	0.125	4	0.25	0.002
CN21	0.25	0.125	0.06	0.5	8	8	0.5	0.5	0.25	1	0.25	1	0.25	0.125	1	0.032
CN22	0.5	0.25	0.12	1	16	128	1	2	0.25	0.5	0.25	0.5	0.25	0.125	1	0.016
CN23	2	0.5	0.03	16	32	64	2	4	0.25	0.5	0.25	0.5	0.25	0.125	1	0.032
CN25	0.12	0.25	0.03	0.5	16	32	0.5	0.5	0.25	0.5	0.25	0.5	0.25	0.125	1	0.032
CN26	0.5	0.5	0.03	1	16	64	0.5	0.5	0.25	0.5	0.25	0.5	0.125	0.06	1	0.016

[a] Pen, penicillin; Ceftri, ceftriaxone; Ctax, cefotaxime; Cefpro, cefprozil; Ceftaz, ceftazidime; 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; ceftazidime: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; erythromycin: susceptible, ≤0.25; intermediate, 0.5; resistant, ≥1; azithromycin: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; clindamycin: susceptible, ≤0.25; intermediate, 0.5; resistant, ≥1; ciprofloxacin: susceptible, ≤2; intermediate, 4, resistant, ≥8; grepafloxacin: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; levofloxacin: susceptible, ≤2; intermediate, 4; resistant, ≥8; sparfloxacin: susceptible, ≤0.5; intermediate, 1; resistant, ≥2; trovafloxacin: susceptible, ≤1; intermediate, 2; resistant, ≥4. Because loracarbef, linezolid, and HMR3647 do not have established breakpoints, they are not listed. The breakpoints for ceftazidime, ciprofloxacin, sparfloxacin, 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, the macrolides, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (data not shown) and carried the *mef* gene.

[b] Isolate WA9 contained both the *mef* 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).

highest MICs (MIC ranges, 8 to 16, 16 to 32, and 64 to 128 μg/ml, respectively) (Table 2). Of the 26 isolates, 20 were resistant to erythromycin and azithromycin (MIC, ≥1 μg/ml). One isolate (WA9) was inducibly resistant to the macrolides and clindamycin (MIC, ≥128 μg/ml for both) and carried both the *ermB* and *mef* genes (17, 27). The other 19 isolates carried the *mef* gene and were resistant to macrolides (MIC range, 1 to 8 μg/ml) but susceptible to clindamycin (MIC, ≤0.25 μg/ml). The quinolones, except for ciprofloxacin, had very low MICs. The MICs of linezolid were comparable to those of the quinolones. The new compound HMR3647 had the lowest MICs of any of the antibiotics tested (MIC range, 0.002 to 0.016 μg/ml).

PFGE analysis of the 26 clinical isolates. Among the 26 *S. pneumoniae* isolates, 20 were considered to be genetically related because they had identical or highly related PFGE patterns with two or three enzymes (Table 3). Of these 20 isolates, 6 Washington isolates (WA7, WA8, WA9, WA11, WA12, and WA14) and 2 Alaska isolates (AK15 and AK18) of *S. pneumoniae* had identical PFGE patterns with all three enzymes. Three Washington isolates (WA1, WA3, and WA5) had identical patterns with two enzymes and had highly related PFGE patterns with one enzyme (*Apa*I). Two Washington isolates (WA2 and WA4) had highly related PFGE patterns with *Sac*II and identical patterns with the other two enzymes. Two Washington isolates (WA10 and WA13) and one Alaska isolate (AK17) had identical PFGE patterns with one enzyme (*Sac*II) and highly related patterns with the other two enzymes. One Alaskan (AK16) and three Canadian (CN19, CN20, and CN24) isolates had related PFGE patterns for each of the three enzymes.

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 *Apa*I and *Sac*II enzymes (Fig. 1) and a three-band difference for the *Sma*I PFGE pattern compared to the *Sma*I PFGE pattern of isolate WA8.

IS*1167* RFLP analysis. Of the 20 isolates with the same or related PFGE patterns, restriction fragment length patterns

TABLE 3. Restriction enzyme PFGE patterns[a]

Isolate	Isolation date (mo/yr)[b]	Origin	Pattern with enzyme:		
			*Sma*I	*Sac*II	*Apa*I
Related isolates					
WA8	12/95	WA	S26	C8	A37
WA9	12/95	WA	S26	C8	A37
WA11	1/96	WA	S26	C8	A37
WA12	4/96	WA	S26	C8	A37
WA14	6/96	WA	S26	C8	A37
WA7	3/97	WA	S26	C8	A37
AK15	4/94	AK	S26	C8	A37
AK18	?/97	AK	S26	C8	A37
WA3	2/97	WA	S26	C8	$A37_2$
WA5	3/97	WA	S26	C8	$A37_2$
WA1	2/97	WA	S27	C8	$A37_2$
WA2	2/97	WA	S26	$C8_2$	A37
WA4	3/97	WA	S26	$C8_2$	A37
WA10	12/95	WA	S26	$C8_1$	$A37_1$
WA13	6/96	WA	S26	$C8_1$	$A37_1$
AK17	2/96	AK	$S26_1$	$C8_1$	A37
AK16	8/94	AK	$S26_3$	$C8_3$	$A37_1$
CN19	4/95	Canada	$S26_3$	$C8_3$	$A37_2$
CN20	4/95	Canada	$S26_3$	$C8_3$	$A37_2$
CN24	1/96	Canada	$S26_3$	$C8_3$	$A37_3$
Unrelated isolates					
CN22	6/95	Canada	$S26_2$	C18	A47
CN23	11/95	Canada	$S26_2$	C19	A49
WA6	2/97	WA	S28	C9	A38
CN21	5/95	Canada	S35	C17	A46
CN25	1/95	Canada	S36	C20	A48
CN26	4/95	Canada	S37	C21	A50
Multiresistant clones					
SP27 (267-Spain-23F-1)		Spain	$S26_4$	C10	A39
SP28 (681-Spain-6B-2)		Spain	S29	C11	A40
SP29 (665-France-9V-3)		France	S30	C12	A41
SP30 (17219-S. Africa-19A-7)		S. Africa	S31	C13	A42
SP31 (50803-S. Africa-6B-8)		S. Africa	S32	C14	A43
SP32 (51702-S. Africa-19A)		S. Africa	S33	C15	A44

[a] Each *Apa*I, *Sac*II, and *Sma*I PFGE pattern was assigned a letter and number designation (A 26 to A 50, C 8 to C 21, and S 26 to S 37). PFGE patterns have previously been assigned to isolates from other areas in the United States (reference 23 and unpublished data). Isolates having patterns which differed from these patterns by one to three bands were considered closely related and grouped into subtypes defined by subscripts ($A26_1$, $A26_2$, $C8_1$, etc.). Subscript 1 means one band added to the pattern, subscript 2 means one band deleted from the pattern, subscript 3 means two bands added or deleted, and subscript 4 means a three-band difference (23, 31). WA, Washington; AK, Alaska; S. Africa, South Africa.

[b] ?, month unknown.

were either identical or had a one-band difference in the IS*1167* RFLP patterns. The six clone isolates (SP27 to SP32) had unique IS*1167* RFLP patterns distinctly different from that of the Washington clone (data not shown). The remaining Washington isolate (WA6) and the Canadian isolates (CN21, CN22, CN23, CN25, and CN26) had six- to nine-band differences in patterns from the Washington clone (data not shown).

***mef* RFLP analysis.** All 20 isolates with the same or related PFGE patterns had identical *mef* RFLP patterns and were distinct from unrelated *S. pneumoniae* isolates that carried the *mef* gene (Fig. 2). The unrelated Washington isolate (WA6) and the unrelated Canadian isolates (CN21, CN22, CN23, CN25, and CN26) had distinct RFLP patterns having one to five bands with more than one band different from the Washington clone (data not shown). In contrast, none of the clone isolates (SP27 to SP32) carried the *mef* gene, and RFLP analysis could not be performed.

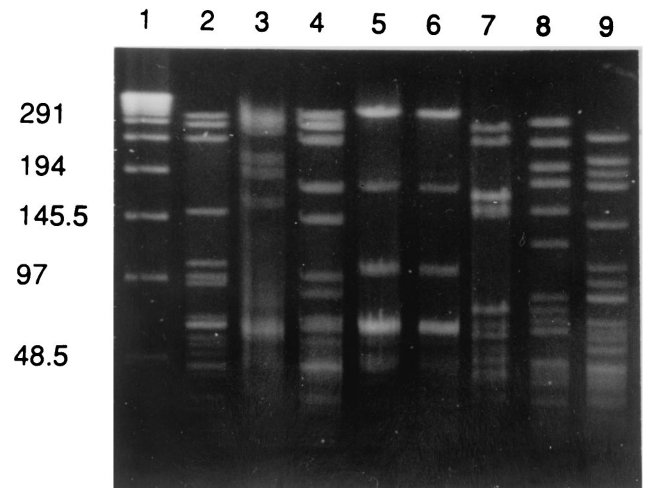

FIG. 1. PFGE after *Apa*I restriction digestion. Lane 1, lambda ladder; lane 2, Washington isolate WA8; lane 3, 19F isolate from CDC; lane 4, 23F isolate from Spain; lanes 5 and 6, 19A isolates from South Africa; lane 7, 6B isolate from Spain; lane 8, 6B isolate from South Africa; lane 9, 9V isolate from France. The numbers on the side represent molecular weight standards in kilobases.

DISCUSSION

PFGE analysis using three different enzymes and IS*1167* and *mef* RFLP typing identified a unique, multiresistant, serogroup 19 *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, *Apa*I (Fig. 1) and *Sac*II, and in IS*1167* RFLP typing, indicating that it was completely different from the Washington clone. In previous work with *S. pneumoniae* and *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 *S. pneumoniae* isolates. In addition, the new compound HMR3647 may offer another therapeutic choice in the future.

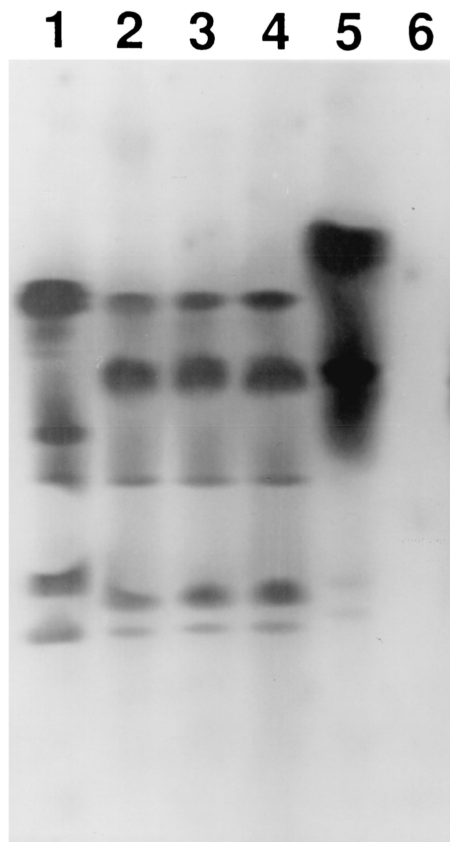

FIG. 2. Hybridization of *Hin*dIII-digested whole DNA with *mef* probe. Whole DNA was digested with *Hin*dIII and run on a 0.7% agarose gel for 3 h. The *mef* probe is oligonucleotide probe MF5. Lane 1, 02J1048; lane 2, WA4; lane 3, WA8; lane 4, WA12; lane 5, n011; lane 6, 915. Isolates 02J1048 (*mef* gene positive), n011 (*mef* gene positive), and 915 (*mef* gene negative) are unrelated *S. pneumoniae* isolates.

The Washington clone was identified in both serotype 19F and serotype 19A isolates. Previous reports have identified capsular transformation of the Spanish clone (267-Spain-23F-1) from serotype 23F to 19F (6, 7, 20). Therefore, the presence of both serotype 19F and serotype 19A may reflect capsular transformation within serogroup 19. The 23-valent pneumococcal polysaccharide vaccine contains both serotype 19F and serotype 19A and is an underutilized prevention tool for multiresistant *S. pneumoniae* illness (4, 21). All three of the initial patients, including both fatal cases, were eligible to receive the vaccine because of underlying conditions (i.e., cancer and cardiovascular disease). However, there was no documentation that any of the patients had been vaccinated.

Most of the previous work by others to characterize *S. pneumoniae* clones by PFGE has used only one enzyme and has allowed up to five-band differences in banding patterns (18). Our laboratory used three enzymes, a three-band difference limit, and the requirement that two enzymes give identical or related PFGE patterns. Our laboratory used a one-band difference limit for both the IS*1167* and the *mef* RFLP patterns (22, 31). Klugman and others have proposed that at least two molecular methods be used in identifying clones, as the PFGE with only one enzyme was not sufficient (27; K. Klugman, Letter). In this study, the PFGE and IS*1167* and *mef* RFLP methods correlated well. This is the first demonstration that *mef* can be used for RFLP analysis of *S. pneumoniae*.

A recent report from Spain has described a serogroup 19 isolate that is nonsusceptible to penicillin and resistant to cefotaxime (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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