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Genetic Relatedness within *Streptococcus pneumoniae* Serotype 19F and 23F Isolates in Korea by Pulsed-Field Gel Electrophoresis

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The genetic relatedness of multidrug-resistant pneumococcal isolates of serotypes 19F and 23F was investigated. The DNA fragments digested with *Sma* I were resolved by pulsed-field gel electrophoresis (PFGE). PFGE analysis of 36 *S. pneumoniae* isolates showed 13 different patterns. Among 22 isolates of serotype 19F, 9 different PFGE patterns were present and 14 isolates of serotype 23F isolates represented 5 distinct PFGE patterns. Two isolates of serotype 19F and six isolates of serotype 23F shared the same PFGE pattern (Pattern I). Based on the genetic relatedness within the strains (one genetic cluster was defined as having more than 85% homology), we divided the pneumococcal strains into 6 genetic clusters (I, II, III, IV, V, and VI). The 22 strains of serotype 19F belonged to five distinct genetic clusters (I, II, III, IV, V and VI) and 14 strains of serotype 23F represented two genetic clusters (I and II). These results showed that strains of serotype 19F are genetically more diverse than those of serotype 23F. Serotype 19F isolates with PFGE patterns H and I appeared to be less related to those of the remaining PFGE patterns (A to G) (less than 60% genetic relatedness), but those strains were genetically closely related with serotype 23F. These results suggest that the latter isolates originated from horizontal transfer of the capsular type 19F gene locus to 23F pneumococcal genotypes. In conclusion, the multidrug-resistant pneumococcal isolates of serotype 19F and 23F isolated in Korea are the result of the spread of a limited number of resistant clones.

Key words: pulsed-field gel electrophoresis, PFGE, serotype 19F, serotype 23F, multi-drug resistance

Streptococcus pneumoniae is a part of the normal microflora of the nasopharynx and also an important pathogen that colonizes the upper respiratory tract, thereby causing pneumonia, meningitis, bacteremia, otitis media, and sinusitis (Tuomanen *et al.*, 1995). To date, *S. pneumoniae* is classified into more than 90 distinct serotypes based on the differences in antigenicity of the capsular polysaccharides (Robbins *et al.*, 1983). The distri- bution of serotypes varies in different populations and geographic areas, and some pneumococcal serotypes are known to be more virulent than other types (Reinert *et al.*, 1995). In the 1990s, the emergence and rapid spread of high-level penicillin-resistant and multidrug-resistant pneumococci has become a major therapeutic threat (Appelbaum, 1992). In particular, penicillin-resistant *S. pneumonaie* belongs predominantly to serotypes 6B, 14, 19F, 23F, and 9V, which are commonly associated with invasive diseases (Ferroni *et al.*, 1996; Kaplan, 1996).

Based on our previous study for pneumococci collected during 1996~1999, penicillin-nonsusceptible (76.3%) and multidrug-resistant (89.4%) pneumococci were highly prevalent in Korea (unpublished observations) and serotypes 19F and 23F are the most common and highly associated with penicillin-resistant and multidrug-resistant. Many molecular studies have shown that there has been an intercontinental spread of penicillin-resistant pneumococci of serotypes 23F, 6B, 14, and 9V in many parts of the world (Hermans *et al.*, 1997; Reichmann *et al.*, 1995; Munoz *et al.*, 1991).

In this study, we have used pulsed-field gel electrophoresis (PFGE) to conduct a molecular analysis of multidrug-resistant pneumococcal isolates of serotypes 19F and 23F in Korea. In addition, we have examined the genetic relationship between serotype 19F and 23F pneumococcal isolates.

Materials and Methods

Bacterial isolates and culture conditions

We used clinical isolates of *S. pneumoniae*, collected between 1996 and 1999 from the patients who were admitted to the university hospitals in Seoul. They included 22 isolates of serotype 19F and 14 isolates of serotype 23F, with all isolates being penicillin-resistant and multidrug-resistant. The clinical sources and relevant properties of all the strains used in this study were

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Table 1. Properties of serotype 19F and 23F clinical isolates of *S. pneumoniae* used in this study.

Serotype	Strain No.	Source	Antibiotic resistance pattern[b]	PFGE pattern
19F	KNIH1021	sputum	CEF, CLN, ERY, PEN, TET, T-S	A
19F	KNIH1053	sputum	CLN, ERY, PEN, T-S	A
19F	KNIH1075	sputum	CLN, ERY, PEN, TET, T-S	A
19F	KNIH1083	sputum	CLN, ERY, PEN, TET, T-S	A
19F	KNIH1087	sputum	CLN, ERY, PEN, TET, T-S	A
19F	KNIH1109	sputum	CLN, ERY, PEN, TET, T-S	A
19F	KNIH1137	sputum	CLN, ERY, PEN, TET, T-S	A
19F	KNIH2031	CSF[a]	CLN, ERY, PEN, TET, T-S	A
19F	KNIH2027	throat	CEF, CHR, CLN, ERY, PEN, TET, T-S	B
19F	KNIH1060	sputum	CLN, ERY, PEN, TET, T-S	C
19F	KNIH1119	sputum	CEF, CLN, ERY, PEN, TET, T-S	C
19F	KNIH1121	sputum	CEF, CLN, ERY, PEN, T-S	C
19F	KNIH1130	sputum	CLN, ERY, PEN, TET, T-S	C
19F	KNIH1151	sputum	CLN, ERY, PEN, TET, T-S	C
19F	KNIH1156	blood	CEF, CLN, ERY, PEN, TET, T-S	C
19F	KNIH1133	blood	CLN, ERY, PEN, T-S	D
19F	KNIH1139	sputum	CLN, ERY, PEN, TET, T-S	E
19F	KNIH2033	CSF	CHR, CLN, ERY, PEN, TET, T-S	F
19F	KNIH1154	blood	CLN, ERY, PEN, TET, T-S	G
19F	KNIH1059	sputum	CHR, ERY, PEN, TET, T-S	H
19F	KNIH1042	blood	CLN, ERY, PEN, TET, T-S	I
19F	KNIH1100	sputum	CHR, ERY, PEN, TET, T-S	I
23F	KNIH1065	blood	CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH1084	sputum	CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH1091	sputum	CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH1127	sputum	CEF, CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH1131	throat	CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH2034	CSF	CHR, CLN, ERY, PEN, TET, T-S	I
23F	KNIH1103	blood	CHR, CLN, ERY, PEN, TET, T-S	J
23F	KNIH1066	sputum	CEF, CLN, ERY, PEN, TET, T-S	K
23F	KNIH1155	blood	CLN, ERY, PEN, TET, T-S	L
23F	KNIH2005	throat	CLN, ERY, PEN, TET, T-S	M
23F	KNIH2006	throat	CLN, ERY, PEN, TET, T-S	M
23F	KNIH2008	throat	CHR, CLN, ERY, PEN, TET, T-S	M
23F	KNIH2018	throat	CHR, CLN, ERY, PEN, TET, T-S	M
23F	KNIH2019	throat	CHR, CLN, ERY, PEN, TET, T-S	M

[a]Cerebrospinal fluid
[b]CEF, cefotaxime; CHR, chloramphenicol; CLN, clindamycin; ERY, erythromycin; PEN, penicillin; TET, tetracyclin; T-S, trimethoprim-sulfamethoxazole

described in Table 1. Pneumococci were grown on blood agar plates (Difco, USA) containing 5% defibrinated sheep blood at 37°C in 5% CO_2 for 18 h.

Serotyping and antimicrobial susceptibility testing

Pneumococci were serotyped on the basis of capsular swelling (Quellung reaction) observed microscopically after suspension in pool-, group-, and type-specific antisera purchased from Statens Seruminsititut (Copenhagen, Denmark) (Facklam and Washington, 1991). Susceptibility testing was performed by the standard broth microdilution method with cation-adjusted Muller-Hinton broth (Difco) supplemented with 3% lysed horse blood, as described by the National Committee for Clinical Laboratory Standards (NCCLS, 1999). Strains were defined as antibiotic susceptible, intermediately resistant, or resistant in accordance with NCCLS guidelines (NCCLS, 1997). The quality control strains *S. pneumoniae* ATCC 49619 and *Staphylococcus aureus* ATCC 29213 were included in each run.

DNA preparation and restriction enzyme digestion

Bacteria were grown on blood agar plates (Difco) supplemented with 5% sheep blood for 22 h at 37°C in 5% CO_2. Bacteria cells were washed with 1ml of washing buffer (10 mM Tris, 1M NaCl, pH 8.0) and resuspended

in 0.5×TBE (45 mM Tris, 45 mM boric acid, 1 mM disodium EDTA, pH 8.0). The bacterial suspension was warmed to 50°C and mixed with an equal volume of prewarmed 1.2% low-melting-point agarose (Bio-Rad, USA) prepared in 0.5×TBE. The molten mixture was poured into Plexiglas molds (Bio-Rad) and solidified at 4°C for 20 min. The bacteria embedded in the agarose plugs were lysed by incubation in 1 ml of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) supplemented with 1μg/ml proteinase K and 1% sodium dodecyl sulfate at 50°C for 24 h. Plugs were washed six times in 2 ml of TE buffer at room temperature for 36 hr with gentle agitation. For digestion of DNA, the plugs were equilibrated with 1×restriction buffer at 4°C for 1 h and digested with 40U *Sma* I (Promega, USA) at 25°C overnight.

Pulsed-field gel electrophoresis (PFGE) and dendrogram

The digested DNA plugs were placed in each well of a 1.2% agarose gel prepared in 0.5×TBE and electrophoresed in a contour-clamped homogeneous electric field apparatus (CHEF-DR; Bio-Rad). The PFGE for *Sma* I-digested plugs was performed for 22 h at 6 V/cm in a 0.5×TBE with pulse times from 1 sec to 25 sec. Gels were stained with ethidium bromide solution (1μg/ml) for 30 min and destained in distilled water for 3 h. Å lamda DNA ladder (Roche Diagnostics Co.) was used as a molecular size marker. PFGE patterns were analyzed with Tenover's categorization (Tenover *et al.*, 1995) and comparison of the banding patterns was performed by the unweighted pair group method of average linkage (UPGMA).

Results

Characteristics of 19F and 23F isolates

S. pneumoniae of serotype 19F and 23F, which is the most predominant and highly associated with penicillin resistance in Korea, was analyzed in this study. Table 1 summarizes the relevant characteristics of 22 isolates of 19F and 14 isolates of 23F, including serotype, source, antibiotic resistance pattern, and assignment PFGE pattern. All 36 strains in this study are resistant to penicillin, erythromycin, and trimethoprim-sulfamethoxazole, and, also, all but two of these 36 strains are resistant to clindamycin and all but three strains to tetracyclin. All the isolates of serotypes 19F and 23F were multiple-resistant to three or more of the antibiotics tested. Among various resistance patterns, the resistance to clindamycin, erythromycin, penicillin, tetracyclin, and trimethoprim-sulfamethoxazole was the most predominant in 19F serotypes (12 of 22 strains) and the resistance to chloramphenicol, clindamycin, erythromycin, penicillin, tetracyclin, and trimethoprim-sulfamethoxazole was the most prevalent in 23F serotypes (9 of 14 strains).

PFGE analysis of pneumococcal DNA

Chromosomal DNA was digested with *Sma*I, generating about 11 to 17 fragments ranging from 20 to 400 kb (Fig. 1). The 36 *S. pneumoniae* strains presented 13 different PFGE patterns, designated Pattern A to M, respectively (Table 1). Of these, 9 PFGE patterns were identified among 22 isolates of serotype 19F, and both Pattern A and C accounted for 63.6% of serotype 19F strains (Fig. 2). In addition, among 14 isolates of serotype 23F, 5 PFGE pat-

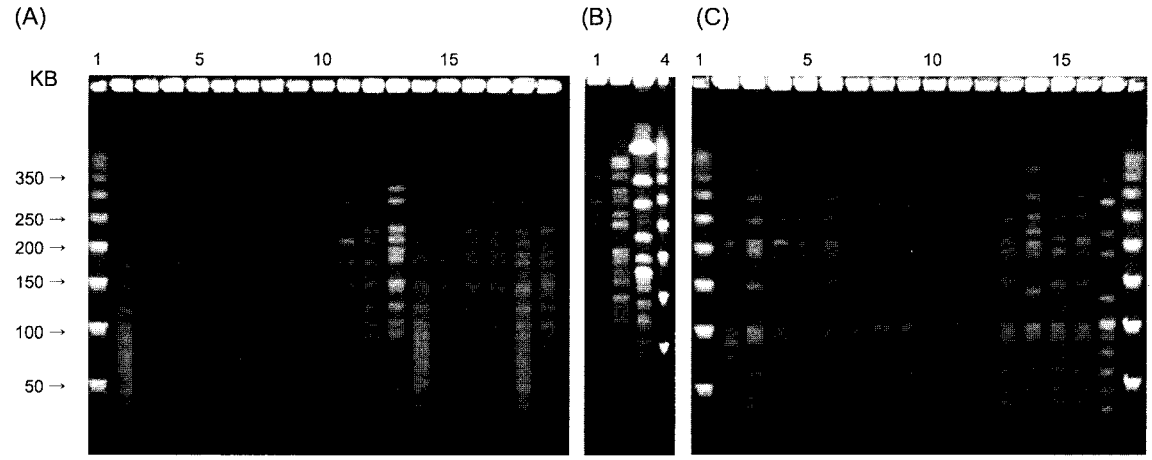

Fig. 1. Pulsed-field gel electrophoresis (PFGE) of *Sma*I restriction fragments of *S. pneumoniae* isolates of capsular serotype 19F and 23F (Korea). (A) Lane 1, PFGE Marker (Roche Diagnostics); lane 2, KNIH1021(19F); lane 3, KNIH1053(19F); lane 4, KNIH1075(19F); lane 5, KNIH1083(19F); lane 6, KNIH1087(19F); lane 7, KNIH1109(19F); lane 8, KNIH1137(19F); lane 9, KNIH1139(19F); lane 10, KNIH1042(19F); lane 11, KNIH2027(19F); lane 12, KNIH2031(19F); lane 13, KNIH1156(19F); lane 14, KNIH1060(19F); lane 15, KNIH1119(19F); lane 16, KNIH1121(19F); lane 17, KNIH1130(19F); lane 18, KNIH1151(19F); lane 19, KNIH1154(19F). (B) Lane 1, KNIH1133(19F); lane 2, KNIH2033(19F); lane 3, KNIH1150(19F); lane 4, PFGE Marker (Roche Diagnostics).

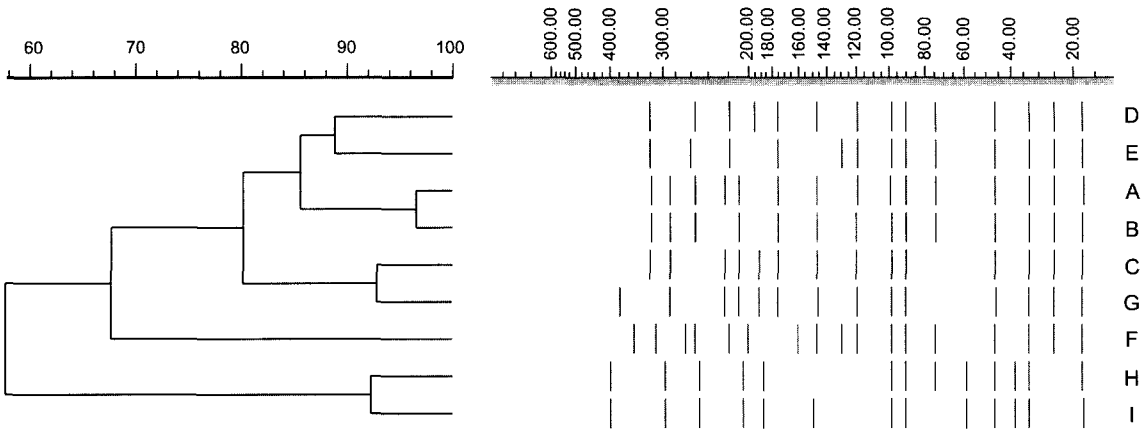

Fig. 2. Dendrogram of the PFGE patterns of 22 penicillin-nonsusceptible pneumococcal serotype 19F isolates. The scale measures similarity values, and the letters at the right indicate different PFGE patterns.

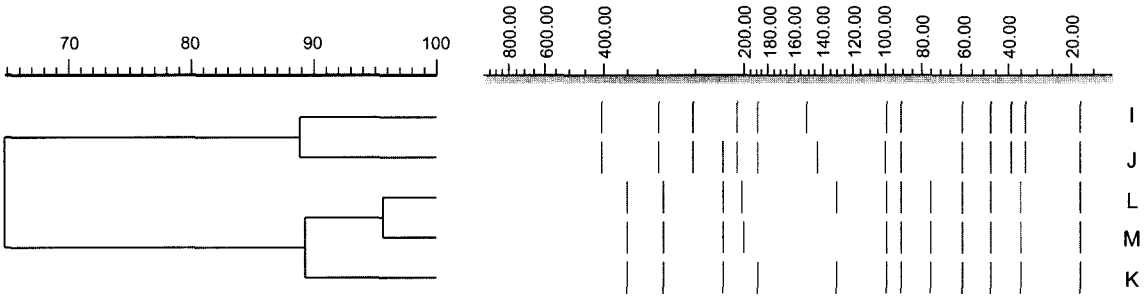

Fig. 3. Dendrogram of the PFGE patterns of 14 pencillin-nonsusceptible pneumococcal serotype 23F isolates. The scale measures similarity values, and the letters at the right indicate different PFGE patterns.

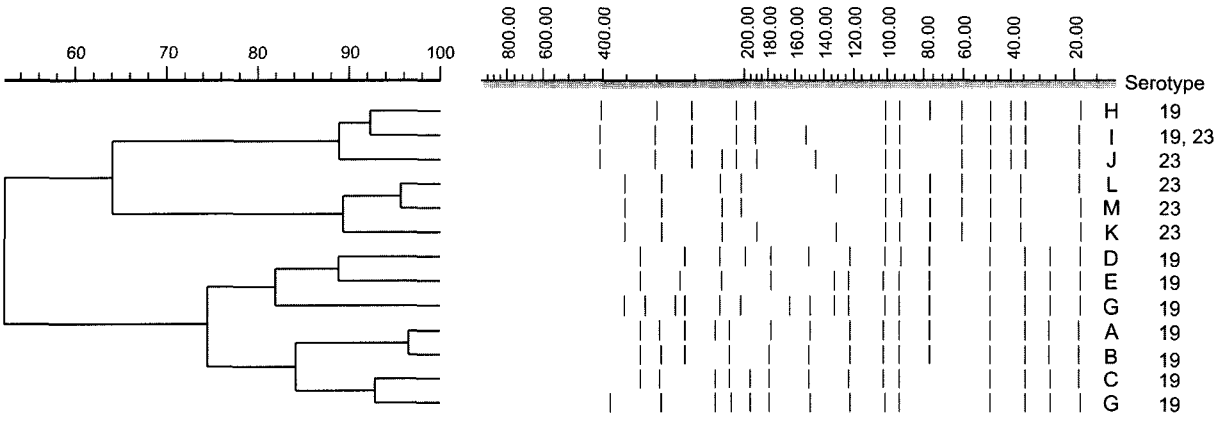

Fig. 4. Genetic relatedness within 36 penicillin-nonsusceptible pneumococcal serotype 19F and 23F isolates. Codes and refer to genetic clusters of pneumococcal strains.

terns was found and 11 strains (78.6%) belonged to the two predominant pattern (Pattern I and M) (Fig. 3). Notably, two isolates of serotype 19F and six isolates of serotype 23F shared the same PFGE pattern (Pattern I), which was the most common pattern in serotype 23F isolates.

Cluster analysis of PFGE results

A dendrogram was constructed by computer analysis of the DNA fingerprints (Fig. 2, 3, and 4). The isolates segregated into 6 genetic clusters (I, II, III, IV, V, and VI) based on more than 85% homology as the criteria of a cluster (Fig. 4). The size of clusters varied from 1 to 9 strains; Cluster I (7 strains of 23F serotype and 2 strains of 19F serotype), Cluster II (6 strains of 23F serotype), Cluster III (2 strains of 19F serotype), Cluster VI (1 strain of 19F serotype), Cluster V (9 strains of 19F serotype),

and Cluster VI (7 strains of 19F serotype). Two clusters (I and II) were found among 23F isolates and five distinct clusters I (I, III, VI, V, and VI) were among 19F isolates, which were more heterogeneous than 23F isolates. Particularly, in cluster I, there were three PFGE patterns (H, I, and J), which were closely related with each other in spite of different serotype and appeared to be less related to the remaining PFGE patterns (A to G) of serotype 19F (less than 60% genetic relatedness) (Fig. 2).

Discussion

Penicillin-resistance and multidrug-resistance among pneumococcal strains have rapidly spreaded throughout the world since the 1980s (Appelbaum, 1992; Ferroni *et al.*, 1996; Reinert *et al.*, 1995; Hermans *et al.*, 1997; Munoz *et al.*, 1991; Reichmann *et al.*, 1995). Based on the reports from a few investigators in Korea during the 1990s, penicillin-nonsusceptible *S. pneumoniae* ranged approximately from 70 to 80%, which is relatively high compared with other countries (Kim *et al.*, 1996; Song *et al.*, 1999).

We determined the serotype distribution and susceptibility to various antibiotics of 142 *S. pneumoniae* isolated in Korea (unpublished observations). Our study showed that four serotypes (19F, 23F, 14, and 9V) are the most predominant, accounting for 50.7% of all the isolates. Also, 76.8% of the 142 isolates are resistant to penicillin and 72.2% of 126 penicillin-nonsusceptible isolates are associated with seven serotypes (19F, 23F, 9V, 6A, 14, 11A, and 6B). Therefore, we examined the genetic relatedness within pneumococcal serotype 19F and 23F isolates, which are all penicillin-resistant strains and the most predominant serotypes among the pneumococci collected from patients in the our study.

For isolates of the same serotype, PFGE classified several isolates that were not distinguished by serotype. However, some isolates of different serotypes showed more than 80% homology, and this result suggests that these isolates may have initially derived from the same clonal strain. Thus, we found that serotype was not closely linked to the genetic lineage of the isolate and PFGE typing have been useful for distinguishing pneumococcal isolates in the same serotype.

By using a cutoff value of 85% homology, 5 different clusters were found among the 19F isolates and 2 different clusters were among the 23F isolates, respectively, suggesting clonal diversity among this serotype. However, Pattern A and C were the most common among 19F isolates and Pattern I and M, among 23F isolates, respectively and this result suggests that the emergence of multidrug-resistant 19F and 23F isolates is due to the spread of a few predominant clones.

Interestingly, in spite of different serotype, a genetic relationship has been observed between serotype 19F and 23F strains. These strains, included in Cluster I, showed the identical or very close PFGE pattern. Particularly, two 19F strains (KNIH1042 and KNIH1100) exhibited the PFGE pattern identical to that found in 23F isolates, suggesting horizontal gene transfer of the capsular type 19F gene locus to 23F pneumococci. A high frequency of capsular exchange has been reported in molecular epidemiological studies of penicillin-resistant isolates from many countries (Nesin *et al.*, 1998). In addition, even though strains shared a common PFGE background, they expressed different antibiogram. The relationship between antibiotic susceptibility and PFGE patterns was independent.

In conclusion, pneumococcal strains belonging to serotype 19F and 23F display genetic diverse within a single serotype. Serotype 19F pneumococcal isolates are genetically more diverse than 23F isolates. These results suggest that the prevalence of multidrug-resistant 19F and 23F pneumococci is the result of the spread of a limited number of resistant clones.

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