Genetic Diversity and Exotoxin A Production of Group A Streptococci Causing Sepsis

The M protein and streptococcus pyrogenic exotoxin (SPE A) are important virulence factors in group A streptococci (GAS) infections. The *emm* types of GAS strains isolated from patients with sepsis were determined by sequencing the 5′ N-terminus of the *emm* gene, encoding the M protein, and clonality analysis using pulsed-field gel electrophoresis. The presence of *speA* and production of SPE A were also examined. There were no predominant GAS clones. The *emm* genotypes were variable, and the most common genotype was *emm*13 (17.9%). The production prevalence of SPE A was 21.4%. The low mortality rate (7.1%) of GAS sepsis might be attributable to the low incidence of virulent strains such as *emm*1 (10.7%) and *emm*3 (7.1%), as well as to low production rate of SPE A.

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INTRODUCTION

There has been an increase in the number of reported cases of group A streptococci (GAS) sepsis recently (1). The number of severe GAS infections, such as toxic shock-like syndrome (TSLS), also has increased in many countries, reflecting changing epidemiology and clinical patterns of invasive streptococcal infections (2-5). International communications about disease and strain typing are important in defining and monitoring trends in infectious diseases (6). The M protein and streptococcus pyrogenic exotoxin (SPE A) are the most important virulence factors in GAS infections. An increasing frequency of M serotypes 1 and 3 has been observed in the United States (7) and Europe (8). These types are more often involved in invasive and fatal infections than are other M serotypes (2, 9).

Although M typing has long been used for epidemiologic purpose, it is difficult to perform routinely because it is hard to produce and maintain more than 80 kinds of anti-M sera. Recently, genetic typing of GAS became available through the sequencing of the 5′ N-terminal end of the *emm* gene, which encodes the M protein (10, 11). However, *emm* typing might not adequately reflect the genetic diversity of bacterial strains, as suggested by the finding that strains expressing the same M serotype can be distinguished by pulsed-field gel electrophoresis (PFGE) (6, 12).

In this work, genetic variability of GAS strains isolated from patients with sepsis was determined by *emm* genotyping and PFGE. Restriction-fragment length polymorphism (RFLP) analysis of *emm* PCR products was applied for the simple discrimination of each strain. The presence of *speA* and the production of SPE A were also examined to estimate the positive rate of this toxin in GAS sepsis.

MATERIALS AND METHODS

Bacterial strains

A total of 28 GAS strains isolated from blood cultures from 1992 through 1999 were collected from four university hospitals: Seoul National University Hospital (17 strains), Asan Medical Center (4 strains), Ewha University Mokdong Hospital (1 strain), all located in Seoul, and Gyeongsang National University Hospital (6 strains), located in Jinju. The medical records were reviewed for all patients, with note taken of age, sex, underlying diseases, the presence of TSLS, and the clinical outcome. There were 28 patients with a median age of 51 yr (range 1 month-74 yr), of whom 38.5% were

15 yr or younger and 23.1% were older than 60 yr. Nine patients had cancer. The sources of sepsis were cellulitis in 4 cases, pneumonia in 3, and peritonitis in 2. In 11, no focus of infection could be identified. All but 2 patients of TSLS survived (Table 1).

For identification of GAS, β-hemolytic colonies on sheep blood agar plate were tested with bacitracin, latex agglutination (Strepto LA kit; Denka Seiken, Tokyo, Japan), and the VITEK GPI card (bioMeriux, Hazelwood, MO, U.S.A.).

T typing

All 28 isolates were tested for T type by slide agglutination (Sevac AS, Prague, Czech Republic).

Table 1. Clinical features of 28 cases of group A streptococcal bacteremia

Strain No.	Isolation time	Hospital	Sex	Age (yr)	Underlying disease	Outcome
SJ-1	96.5	A	M	36	LC, varix bleeding	Alive
SJ-2	96.5	A	M	11	Sepsis, lymphangitis	"
SJ-3	96.7	A	M	59	LC, PLCa	"
SJ-4	96.12	A	F	71	Cervix Ca, psoas abscess	"
SJ-5	97.1	A	M	63	PLCa	"
SJ-6	97.2	A	M	1	Omphalitis	"
SJ-7	97.4	A	F	6	Angina, pharyngitis	"
SJ-8	98.7	A	F	56	Cervix Ca	"
SJ-9	97.5	A	M	42	Chronic hepatitis	"
SJ-10	97.6	A	F	53	Cervix Ca, bacterial meningitis	"
SJ-11	98.4	A	F	11	SLE, FUO	"
SJ-12	95.7	B	F	74	SCLCa, neutropenic fever	"
SJ-13	95.6	B	M	63	Pharyngitis, cellulitis	"
SJ-14	96.11	B	M	51	AGCa, intestinal obstruction	"
SJ-15	95.7	B	M	15	Right foot cellulitis	"
SJ-16	92.3	A	M	9	Spontaneous bacterial peritonitis	"
SJ-17	97.2	C	F	32	Pregnancy, IUFD, TSLS	DoD
SJ-18	95.7	D	M	55	Diabetic foot	Alive
SJ-19		D			Not available	
SJ-20	95.3	D	M	62	Duodenal Ca, peritonitis	Alive
SJ-21	98.9	D	M	56	Cellulitis, TSLS	DoD
SJ-22	95.1	A	M	9	ALL	Alive
SJ-23	96.10	A	M	5	Brain stem glioma, pneumonia	"
SJ-24	97.10	A	F	1	Omphalitis, neonatal sepsis	"
SJ-25	93.1	A	F	1	Pneumonia, neonatal sepsis	"
SJ-26	99.3	A	M	60	Deep vein thrombosis	"
SJ-27	99.1	D	M	57	Diabetic foot, cellulitis	"
SJ-28	99.1	D	M	55	Aspiration pneumonia	"

Hospitals: A, Seoul National University Hospital; B, Asan Medical Center; C, Ehwa Woman's University Mokdong Hospital; D, Gyeongsang National University Hospital.
LC, liver cirrhosis; PLCa, primary liver cancer; SLE, systemic lupus erythematosus; FUO, fever of unknown origin; SCLCa, small-cell lung cancer; AGCa, advanced gastric cancer; IUFD, intrauterine fetal death; DoD, died of streptococcal disease; TSLS, toxic shock-like syndrome; DM, diabetes mellitus; ALL, acute lymphocytic leukemia.

emm genotyping

The *emm* sequence analysis method was modified from that of Beall et al. (11). Forward and backward primers were used in PCR as described previously (12). The product was sequenced using primer 5´-TATTCGCTTAGAAAATTAAAA-ACAGG-3´ (13) with dye-labeled terminator PCR mix and subjected to automated sequence analysis on a 373 Autosequencer (Applied Biosystems, Foster City, CA, U.S.A.). The DNA sequences were subjected to homology searches against the bacterial DNA database with the BLAST program from the U.S. National Center for Biotechnology Information. The corresponding *emm* sequence diverged by less than 4% over 150 to 200 bases.

The *emm* PCR products were cut with *Hae*III and separated on 12% polyacrylamide gel for 2 hr at 15 mA. Gels were stained with ethidium bromide and photographed (Polaroid 667 film) under UV light.

PFGE

Macrorestriction and PFGE were performed as previously described with several modifications (2). Genomic DNA was digested with *Sma*I or *Apa*I for 6 hr at 25°C. Fragments of DNA were separated on 1% pulsed field-certified agarose (Bio-Rad, Hercules, CA, U.S.A.) in 0.5 × TBE buffer using a CHEF-Mapper XA system (Bio-Rad) for 27 hr with a 2.16- to 26.29-sec switch time and 74% ramping factor between 10 and 700 kb at a 120° angle and 6 V/cm. Gels were stained with ethidium bromide, rinsed in distilled water, and photographed under UV light.

Detection of *speA* and SPE A

The PCR primers were used for detection of *spe*A as previously described (16). Initial denaturation of DNA at 94°C for 3 min was followed by 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The amplicon size for *spe*A was 205 bp.

The bacterial suspension was precipitated with ethanol, mixed with loading buffer, and boiled for 5 min. The denatured protein was separated on 10% polyacrylamide gel for 1.5 hr at 16 mA. The gel was transferred to the nitrocellulose membrane for 15 hr at 80 mA, and the membrane was treated with anti-SPE A antibody. Color development was observed with anti-rabbit antibody and 4-chloro-1-naphthol.

RESULTS

There were three (10.7%) strains each of the T1, T12, and T28 types (Table 2). Ten strains (35.7%) were nontypeable. Two strains showed a rare combination of T and *emm* types, namely SJ8 (T6, *emm*13) and SJ21 (T4, *emm*2).

The most frequent *emm* type was 13 (17.9%). The next most common types were *emm*12 (14.3%), *emm*1 (10.7%), and *emm*28 (10.7%). There were 13 *emm*-types (Table 2).

One to three small fragments were separated on the gel after treatment with *Hae*III (Fig. 1A). All strains except *emm*6 were digested with *Hae*III and were classified into seven types (Fig. 1B). The *emm*49, *emm*53, and SP2346 types demonstrated similar RFLP patterns (H1) with *emm*1. The *emm* 2, 22, and 28 types also showed similar RFLP patterns (H2). Otherwise, each *emm* type had a unique RFLP pattern (Table 2).

All but 4 of the 28 strains were digested with *Sma*I, showing 10 to 15 large fragments (Fig. 2A). Two strains of *emm*75, one of *emm*6, and one of *emm*12 were not digested with *Sma*I, whereas they were cut with *Apa*I (Fig. 2B). The *emm*13, 12,

and 1 types showed some variance of PFGE pattern. Otherwise, each *emm* type had a unique PFGE pattern. Thirteen PFGE patterns were found by *Sma*I (Fig. 3). All strains were cut with *Apa*I, and the genetic diversity revealed by *Apa*I agreed with the result of *Sma*I digestion. Exceptionally SJ6 and SJ8 (*emm*13) showed 3 different fragment bands with *Apa*I, while they were the same with *Sma*I enzyme.

The presence of *speA* and SPE A toxin was found only in the *emm*1, *emm*3, and SP2346 strains (Table 2).

DISCUSSION

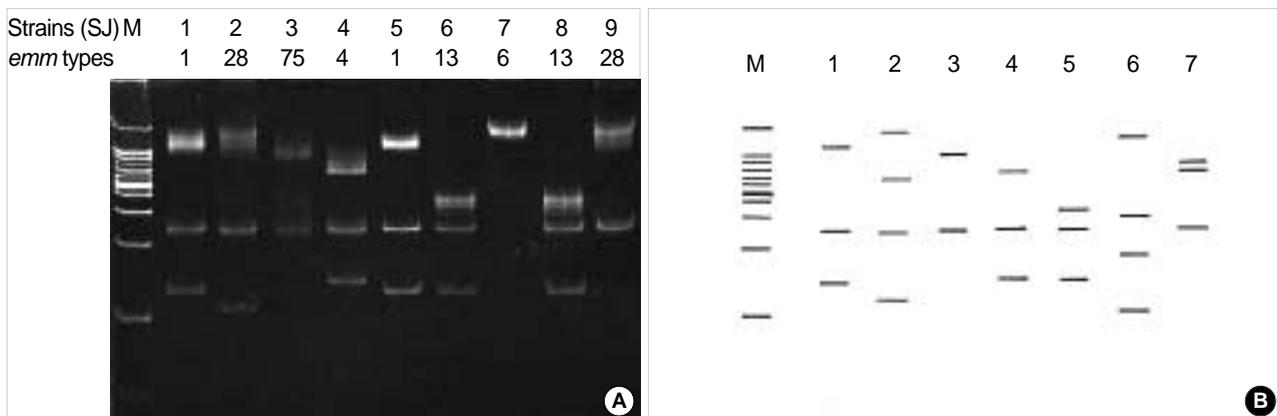GAS have long been recognized as serious human pathogens

Fig. 1. (A) *emm*-RFLP restricted with *Hae*III enzyme runned on 12% acrylamide gel and (B) its schematic pattern. Number of each lane in (B) represents *emm* RFLP pattern. Lane M, molecular marker of 100 bp ladder.
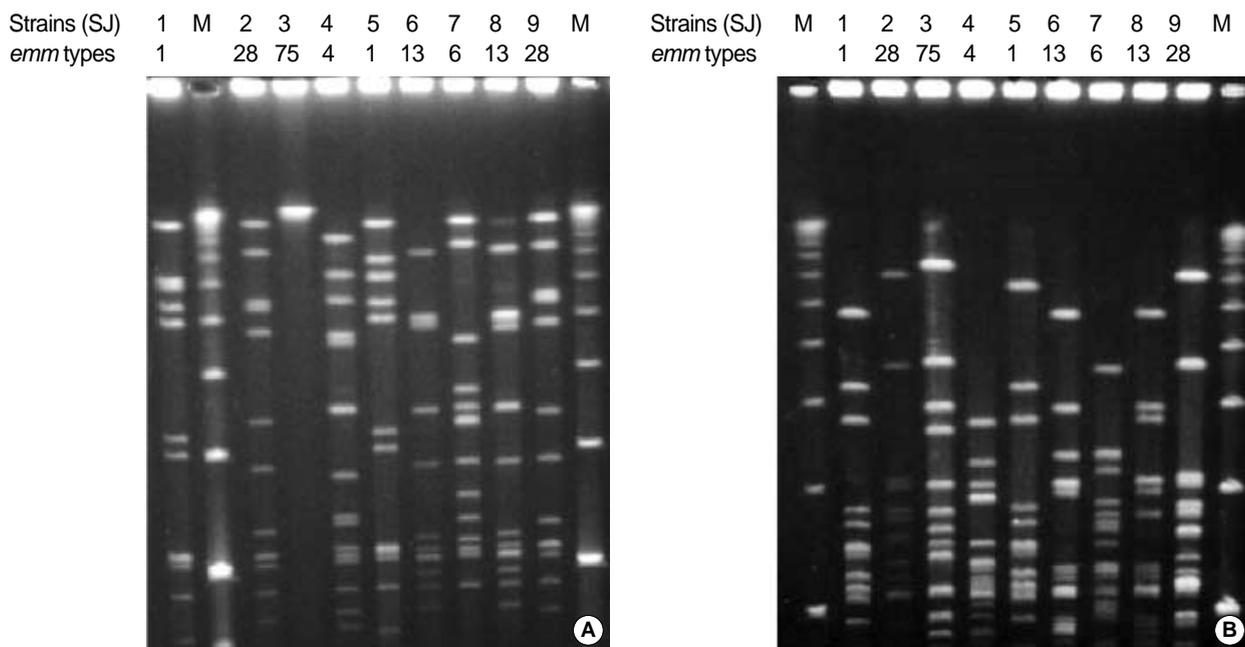

Fig. 2. Genomic DNA fingerprints by pulsed-field gel electrophoresis after digestion with (A) *Sma*I and (B) *Apa*I enzymes. Lane M, molecular marker of low range PFG.

Table 2. T type, *emm* genotype, *emm*-RFLP, PFGE pattern, and presence of *spe*A and production of SPE A of group A streptococci isolated from blood

Strain No.	T type	*emm* genotype	*emm*-RFLP	PFGE pattern	*spe*A gene	SPE A toxin
SJ-1	1	1	H1-a	L1	+	+
SJ-5	1	1	H1-a	L2	+	+
SJ-14	1	1	H1-a	L1	+	+
SJ-21	4	2	H2-c	M	-	-
SJ-12	3	3	H6	A	+	+
SJ-17	3/B3264	3	H6	A	+	+
SJ-4	NT	4	H4	I	-	-
SJ-10	4	4	H4	I	-	-
SJ-7	6	6	Uncut	B	-	-
SJ-19	6	6	Uncut	Uncut	-	-
SJ-15	12/27	12	H7	F3	-	-
SJ-16	12	12	H7	F2	-	-
SJ-26	NT	12	H7	Uncut	-	-
SJ-27	12	12	H7	F1	-	-
SJ-6	NT	13	H5	D	-	-
SJ-8	6	13	H5	D	-	-
SJ-13	NT	13	H5	D	-	-
SJ-20	NT	13	H5	H	-	-
SJ-22	NT	13	H5	D	-	-
SJ-18	12	22	H2-c	G	-	-
SJ-2	28	28	H2-a	C	-	-
SJ-9	28	28	H2-b	C	-	-
SJ-23	28	28	H2-a	C	-	-
SJ-24	NT	49	H1-b	K	-	-
SJ-28	NT	53	H1-b	J	-	-
SJ-3	NT	75	H3	Uncut	-	-
SJ-11	NT	75	H3	Uncut	-	-
SJ-25	B3264	SP2346	H1-b	E	+	+

NT, Nontypeable.

that cause life-threatening illnesses such as sepsis (1), rheumatic fever (RF), necrotizing fasciitis (16), and TSLS (3-5). Sepsis cases attributable to GAS had arisen sporadically in Korea (3, 12). In the U.S.A., 64% of GAS sepsis cases between 1989 and 1990 were caused by M1 serotype, compared with 18% of those before 1979 (17). More than 80% of the GAS isolated from patients with severe disease produce SPE, as opposed to less than 20% of isolates causing less serious disease (17). The M1 and M3 strains may be more likely to cause invasive infections (8). The SPE A protein, an inherent virulence factor, plays an important role in TSLS (5-7, 18). The *emm*1 and *emm*3 strains producing SPE A represented only 17.9% of the isolates in this study, which may explain the good outcomes of sepsis.

Both T and M typing have been used for surveillance and other epidemiologic studies. As anti-T sera have become commercially available, T typing has been widely used for screening for epidemiologic studies of GAS infections (14). We performed T typing, expecting high typeability, as we had already experienced more than 90% typeability for GAS strains isolated from throat cultures (19). However, the proportion of T-nontypeable strains was so high (35.7%) in this
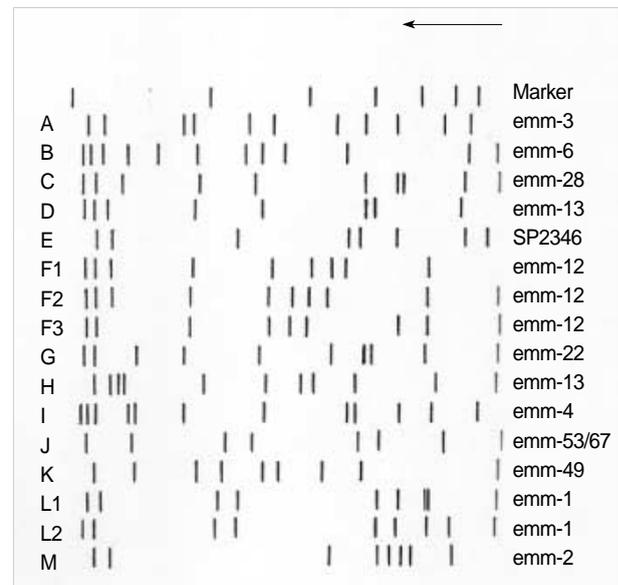

Fig. 3. Schematic pattern of each representative fingerprints produced by *Sma*I enzyme. Marker, molecular marker of low range PFG.

study that T typing was only partly helpful in classifying the isolates. Rare combinations of T and *emm* type were observed in two strains (T6, *emm*13; T4, *emm*2) (14, 20). The M1 and M3 strains are known to have caused the recent outbreaks of RF and TSLS (2, 4-7, 17). Although the study period is different, the previous studies of the prevalence of M types in school children showed that M1 (1.2%) and M3 (4.9%) were rare, while M12 (48.8%) and M5 (14.6%) were very common (n=82) in Seoul. The M1 (3.9%) and M3 (1.3%) strains were likewise rare, while M12 (26.3%), M22 (14.5%), and M28 (10.5%) were frequent (N=76) in Jinju (19). Although *emm*13 was the most common type in this study, it was not found in school children in Seoul and was recovered only from 2.6% of school children in Jinju (19). Another study revealed *emm*1 (20.1%) and *emm*3 (7.7%) were not uncommon in the clinical isolates of GAS in Seoul (12). No *emm*13 was identified among 194 clinical isolates (12). The authors demonstrated the difference of *emm* distribution between school children and the patients with acute pharyngitis in the same area (21). The M types corresponding to *emm*53 and SP2346 have not been identified previously in Korea (19, 22). An *emm* sequence analysis has the advantage of being able to differentiate serologically M-nontypeable strains and to identify new *emm* genes (11).

The *emm*-RFLP technique is easy and economical for epidemiologic studies (15) and is a rapid method of sorting large numbers of diverse GAS into distinct genotypes. The *emm*-RFLP method can be used as an alternative to *emm* sequence typing or PFGE in a small laboratory. The *emm*-RFLP analysis is applicable to areas where GAS are endemic and where the majority of isolates are M nontypeable by serotyping (23).

Simple discrimination of the strains into seven groups was possible by *emm*-RFLP. Identical *emm* restriction cleavage profiles had the same *emm* gene sequences except for a few types. The *emm*49, *emm*53, SP2346 strain could not be differentiated from *emm*1 by *emm*-RFLP, as is also true of *emm*2, *emm*22, and *emm*28. Two strains of the *emm*6 type were not cut with *Hae*III, which suggests the limitation of *emm*-RFLP for epidemiologic studies.

It is notable that the clones of the same *emm* types were maintained over different areas/hospitals for a long periods of time. Although there was no predominant clone, six small clusters of clones (*emm* 1, 3, 4, 12, 13, and 28) were found by PFGE (Table 2). Differences in PFGE patterns were noted within the same *emm*13 type. Two strains of *emm*75 and one each of *emm*6 and *emm*12 were not cut by *Sma*I despite repeated experiments. These strains were, however, digested with *Apa*I, suggesting DNA integrity during the PFGE procedure. Recently, there have been reports that strains carrying the macrolide resistance gene, *mefA*, are not cut with *Sma*I (24, 25). This gene may encode a *Sma*I site-modifying activity (25). Those strains uncut with *Sma*I in this study were confirmed to have the *mefA* gene by PCR (unpublished data).

The *Sma*I digests of chromosomal DNA of two *emm*3 strains appeared to have PFGE profiles identical to that of the PFGE-1 clone found in a study of invasive streptococcal disease in Minnesota (26). Although PFGE-1 clone serotyped M3 predominated in TSLS in the U.S.A. (26) and in Japan (27), it represented only 7.1% of our isolates. Notably one *emm*3 strain (SJ17) caused TSLS in this study. On the other hand, the findings of Chaussee et al. (28) did not support a clonal basis for the resurgence of invasive streptococcal infections by observing the genetic heterogeneity using PFGE. There might be no predominant *emm* type or PFGE pattern, because the GAS strains had been isolated sporadically over several years at four hospitals in this study. Drawbacks of this study are lack of evaluation of invasiveness or virulence of GAS isolates and limited number of cases. Following studies with enough number of cases are needed.

In conclusion, there were several small clusters of clones found among the GAS strains causing sepsis by T typing, *emm*, *emm*-RFLP and PFGE profiles. The *emm* types of the GAS isolated were variable, and the most common type was *emm*13. The production of SPE A was associated with *emm*1 and *emm*3 types. The proportion of SPE A-producing GAS was relatively low, which may explain the good prognosis of GAS sepsis in this study.

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