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Distribution of *emm* genotypes and superantigen genes of *Streptococcus pyogenes* isolated in Japan, 1994–9

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SUMMARY

The purpose of this study was to examine characteristic profiles of *Streptococcus pyogenes* clinical isolates isolated in Japan during 1994–9. Genotyping of the M protein (*emm* typing) revealed that *emm* types 12 and 28 were the most common among 316 isolates. Most of the *emm12* isolates were isolated from mucosa, while *emm58* and *emm89* were from skin. Moreover, the *emm3* isolates were dominant in invasive infections. The distribution of 6 superantigen genes showed that all isolates harboured the *mf* gene and many had the *spe*G gene. Invasive isolates were shown to have the *ssa* gene at a higher rate (76%) than noninvasive (37%). The distribution of superantigens was significantly different between *emm* types, but not between isolation sites. These results suggest that the distribution of *emm* types is related to isolation site, whereas superantigen distribution is related to clinical features of *S. pyogenes* infections.

INTRODUCTION

Group A *Streptococcus pyogenes* (GAS) may cause a variety of acute infections such as pharyngitis, impetigo, erysipelas, septicemia and myositis. Non-suppurative sequelae following GAS infections, especially rheumatic fever and acute glomerulonephritis, are still highly prevalent in developing countries and GAS-mediated diseases remain a major concern worldwide. In Japan, as in other countries, a wide variety of GAS infection cases have been reported [1]. In addition, severe and invasive types of GAS infection, including toxic shock-like syndrome (TSLS) and necrotizing fasciitis, are associated with a high mortality rate.

The M protein is a fimbrial protein located on the cell surface and is considered to be a major virulence determinant of GAS. Serotyping based on the serological specificity of M proteins has been conveniently used as a standard method to classify clinical isolates of GAS for the past 60 years, though several problems

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exist. For example, it is extremely difficult to keep an entire set of specific M-typing antisera, which is a limiting factor when determining type distribution [2]. Recently, a new typing system for GAS based on *emm* encoding of the M protein was developed, and Beall et al. [3, 4] reported that nucleotide sequencing of the hypervariable NH_2-terminal portion of the *emm* gene (*emm* genotyping) was very useful for surveying and monitoring GAS isolate diversity.

GAS produces a number of streptococcal pyrogenic exotoxins (SPEs) that are classified as superantigens, and a recent study revealed that there are more than 10 superantigens or superantigen-like toxin molecules in GAS [5, 6]. Stevens and colleagues [7] considered that TSLS may be caused by SPE-A (encoded by the *speA* gene), however, Hsueh et al. [8] reported that the presence of *speA* did not have an involvement with any particular clinical syndrome found in patients with invasive or noninvasive streptococcal infections. An analysis using the GAS genome database of Oklahoma University revealed the presence of several novel superantigens such as SPE-G and SPE-H [5, 6]. Although correlations between the M type, fibronectin-binding protein [9], and serum opacity factor [10] have been reported, few studies have described such between *emm* types and superantigen genes.

The *emm* genotyping method developed by Beall et al. [3] makes it possible to determine rarely occurring *emm* genes. Thus, we chose this method to better characterize the relationship between the M (*emm*) type and superantigen profiles of 316 clinical GAS isolates isolated in Japan during the 5-year period 1994–9 in relation to the clinical status of patients with GAS infections. Herein, we first present these profiles.

MATERIALS AND METHODS

Bacterial isolates

A total of 316 isolates of GAS, isolated during 1994–9 in Japan, were obtained from Tokyo Women's Medical College, Osaka Prefectural Institute of Public Health, and Saga Prefectural Institute of Public Health. Seventeen were from patients with invasive infections, which were TSLS ($n = 15$) and bacteremia ($n = 2$), while 299 were from those with noninvasive infections. Invasive isolates were obtained from blood ($n = 3$) or an unknown location ($n = 12$). Noninvasive isolates included 216 mucosal and 83 skin isolates, with the mucosal isolates coming from the pharynx ($n = 141$), tonsils ($n = 56$), respiratory tract/chest ($n = 5$), ears ($n = 4$), nose ($n = 4$), vagina ($n = 3$), stool ($n = 2$), and urethra ($n = 1$).

DNA isolation

For genotyping, bacterial DNA from GAS was prepared according to Saarela's method [11] with some modifications. Briefly, a few GAS colonies were grown on a Todd Hewitt (Becton Dickinson, Sparks, MD, USA) agar plate containing 5% sheep blood and 0·2% yeast extract, and then transferred to 50 μl of TE buffer (pH 8·0) (10 mM Tris–HCl, 5 mM EDTA). Bacterial cells were harvested by centrifugation (20 800 ***g***, 3 min), and the supernatant was then removed. Micro-tubes containing the bacterial cells were placed in a microwave oven and heated for 5 min at a power setting of 500 W. The pellets were then resuspended in 50 μl of TE buffer, further incubated at 37 °C for 30 min, and centrifuged to remove debris. The supernatant was stored at 4 °C until use as a template for polymerase chain reaction (PCR) analysis.

Genotyping of GAS

Genotyping of the *emm* gene encoding the M protein was done according to the protocol of the Center for Disease Control and Prevention (CDC, http://www.cdc.gov/ncidod/biotech/strep/protocols.html), with minor modifications. Briefly, amplification was performed in a total volume of 50 μl consisting of 1 μl of template solution and 2·5 U of Taq-DNA polymerase (PE Applied Biosystems, Branchburg, NJ, USA), with primer 1 (5′-TATTC/GGCTTAGAAAATTAA-3′) and primer 2 (5′-GCAAGTTCTCAGCTTGTTT-3′) according to the manufacturer's instructions. After an initial heating at 94 °C for 5 min, the PCR mixtures were immediately cycled 30 times with a 15-sec denaturing step at 94 °C, a 30-sec annealing step at 46 °C, and a 75-sec extension step at 72 °C in a PCR2400 thermal cycler (PE applied Biosystems). After the last cycle, the samples were incubated at 72 °C for 7 min and then stored at 4 °C.

The amplified DNA was purified by a Centri-Sep spin column (PE Applied Biosystems). The 5′ *emm* sequence of the PCR product was sequenced using primer 1 with a dye termination mix and analysed using the CDC database (http://www.cdc.gov/ncidod/biotech/strep/strepblast.html). We consider-

ed an isolate to be within a given *emm* type if it had a $\geqslant 95\%$ identity over the first 160 bases obtained.

Determination of superantigen gene

PCR amplification was performed under optimal conditions in order to detect the superantigen genes with specific primer sets. The primer sets used were as follows: *speA-f*, 5′-GCTCAACAAGACCCCGATCC-3′ and *speA-r*, 5′-TGTAGGCTTTGGATACCATCG-3′ (product size; 393 bp, [12]); *speC-f*, 5′-GACTCTAAGAAAGACATTTCG-3′ and *spe*C-r, 5′-AGTCCCTTCATTTGGTGAGTC-3′ (product size; 540 bp, [12]); *speG*-f, 5′-CTGGATCCGATGAAAATTTAAAAGATTTAA-3′ and *speG*-r, 5′-AAGATTCGGGGGGAGAATAG-3′ (product size; 665 bp, [6]); *speH*-f, 5′-TTGGATCCAATTCTTATTATAATACAAC-3′ and *speH*-r, 5′-GACTCTAAGAAAGACATTTCG-3′ (product size; 632 bp, [6]); *mf*-f, 5′-GAATCTACTTGGATCAAGACGG-3′ and *mf*-r, 5′-CCATCACGATTTGCTTCTAACC-3′ (product size 644 bp, this study result); *ssa*-f, 5′-GGTGTAGAATTGAGGTAATTGGGG-3′ and *ssa*-r, 5′-GCTATAGCTGAAGAGCTCACTGTC-3′ (product size; 839 bp, this study result).

Statistical analysis

All analyses were performed using Fisher's exact test (two-tailed) with the computer application StatView (version 5.0J, SAS Institute Inc, Cary, NC, USA).

RESULTS AND DISCUSSION

Dominant *emm* genotypes in GAS isolates obtained from noninvasive infections, 1994–9

Clinical isolates of GAS were obtained from skin and mucosal surfaces of patients with impetigo, pharyngitis, and invasive infections (see Materials and Methods).

The *emm* genotypes of these clinical isolates were determined by nucleotide sequencing of the 5′ *emm* and the presence of superantigen genes was detected by PCR. Genotype *emm12* was found to be the most common (17·4% of the isolates tested), followed by genotypes *emm28* (16·8%), *emm1* (13·3%), and *emm4* (11·1%) (Fig. 1, Table 1). Eight strains were not included in the *emm* genotypes, and were classified as genotypes *st1135* ($n = 1$), *st2926* ($n = 1$), *st88-25* ($n = 1$), *stns292* ($n = 1$), and *stcmuk16* ($n = 4$: Fig. 1, Table 1) as provisional *emm* types. The provisional isolates known as '*st*' (sequence type) have not yet been validated by all of the CDC Streptococcus Laboratory and reference laboratories (http://www.cdc.gov/ncidod/biotech/strep/emmtypes.html). We found that GAS genotypes *emm1*, *emm12* and *emm28* showed a consistently high occurrence in the 1990s in Japan, while genotype *emm4* increased in the late 1990s. These four *emm* genotypes have also spread globally and are known to have caused pharyngeal infections in the United States [13], New Zealand [14], and Iran [15]. Although genotype *emm28* isolates were occasionally reported in Japan in the 1980s [16], the isolation frequency of this GAS genotype increased during the 5 years examined in the present study.

Occurrence of *emm* genotype in mucosal and skin infections

Genotype *emm28* isolates were most prevalent from mucosal sites (see Materials and Methods) and skin infections. Most of the *emm12* isolates were from mucosa (48 of 52, $P = 0{\cdot}0015$), whereas 7 of 11 *emm58* isolates and 5 of 8 *emm89* isolates were from skin infections ($P = 0{\cdot}017$ and $P = 0{\cdot}045$, respectively: Table 1). Furthermore, genotypes *emm1*, *emm2* and *emm4* were largely found in mucosal infection sites (Table 1). The prevalence of these GAS *emm* genotypes in our study was consistent with that for invasive infection and pharyngeal isolates reported in the United States [17] and Canada [18]. Further, Bessen and coworkers reported that M11 and M58, major strains found in skin infections in our study, tended to be tissue-specific. [19].

Occurrence of *emm* genotype in invasive infections

Clinical isolates from cases with invasive GAS infection ($n = 17$) were classified as genotypes *emm1* ($n = 4$), *emm3* ($n = 5$), *emm12* ($n = 3$), *emm18* ($n = 2$), *emm28* ($n = 1$), *emm44* ($n = 1$), and *emm77* ($n = 1$). The results showed that genotype *emm1* and *emm3* isolates occupied 9 (53%) out of 17 cases. Major invasive isolates in Canada have been shown to be serotypes M1 and M3, which made up 39% of that study sample, while M12 and M28 were present in 8·8% and 5·7%, respectively [20]. A previous study

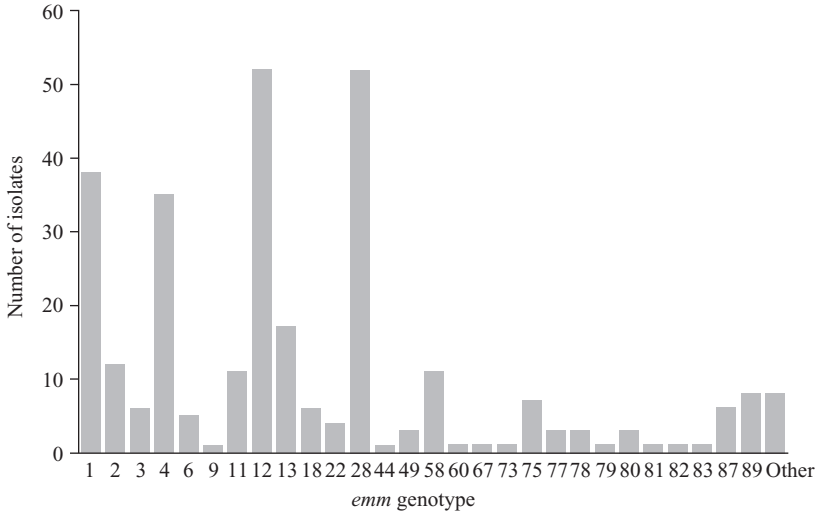

Fig. 1. Distribution of *emm* genotypes in 299 clinical isolates isolated in Japan from 1994–9. Other isolates included the provisional genotypes *st1135*, *st2926*, *st88-25*, *stns292* and *stcmuk16*.

Table 1. *Occurrence of GAS in mucosa and skin specimens among* emm *genotype*

		Isolation site	
emm type	No.	Mucosa	Skin
1	38	33	5
2	12	11	1
3	6	5	1
4	35	27	8
6	5	5	0
11	11	5	6
12	52	48**	4
13	17	11	6
18	6	6	0
28	52	31	21
58	11	4	7**
75	7	5	2
87	6	4	2
89	8	3	5*
Others†	33	18	15
Total	299	216	83

* Fisher's $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.
† Small number of *emm* isolates ($n < 5$) were combined as 'Others'.

suggested that the dominance of the strain with serotype T3 in TSLS was correlated with the increase in this serotype among streptococcal infections [1]. As compared to the distribution of *emm* genotypes in noninvasive infections, the rate of genotype *emm3* strains from invasive infections (29%) was significantly dominant as compared with those from noninvasive infections (2%, $P = 0{\cdot}0003$). However, in other *emm* isolates from invasive infections, we did not find a significantly dominant *emm* genotype, thus, all the strains of the *emm3* genotype were dominant in invasive infections. This is the first known report to show that genotypes *emm44* and *emm77* were found in isolates from invasive GAS infections.

Distribution of superantigens and correlation with clinical status

All isolates possessed the *mf* gene regardless of *emm* genotype or source (Fig. 3), therefore, MF may not be a major virulence factor specific for TSLS or other invasive diseases. MF was previously recognized as a superantigen [21], however, a recent study reported it as a DNase of GAS, and not a mitogen, that stimulates lymphoid cells non-specifically [22]. Moreover, Gerlach et al. [23] showed that MF is not a superantigen.

The *speC* and *speG* genes were seen in more than 70% of the isolates, whereas the *ssa* genes were present in less than 40% (Figs 2, 3, Table 2). Although no correlation between superantigens and isolation sites was found, more of those from the skin possessed the *speG* and *speH* genes than from mucosal sites (Fig. 2). It was also noted that isolates from both the skin and mucosa harboured similar amounts of *speG* (86·7% and 78·2%, respectively) and *speH* (43·3% and 39·8%, respectively).

Although the number of invasive isolates was small, a few interesting superantigen profiles were found in our comparison of invasive and non-invasive isolates. GAS isolates from invasive infections possessed the *ssa* gene at a significantly higher frequency (76%)

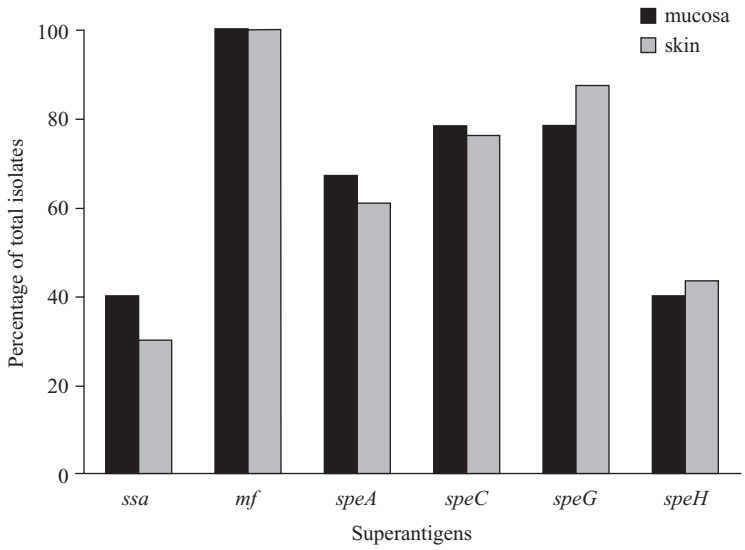

Fig. 2. Prevalence of superantigen genes among GAS isolates from mucosal and skin infections. Mucosal isolates ($n = 216$) from the pharynx ($n = 141$), tonsils ($n = 56$), respiratory tract/chest ($n = 5$), ears ($n = 4$), nose ($n = 4$), vagina ($n = 3$), stool ($n = 2$), and urethra ($n = 1$), as well as skin isolates ($n = 83$) were analysed. No significant differences were observed using Fisher's exact test.

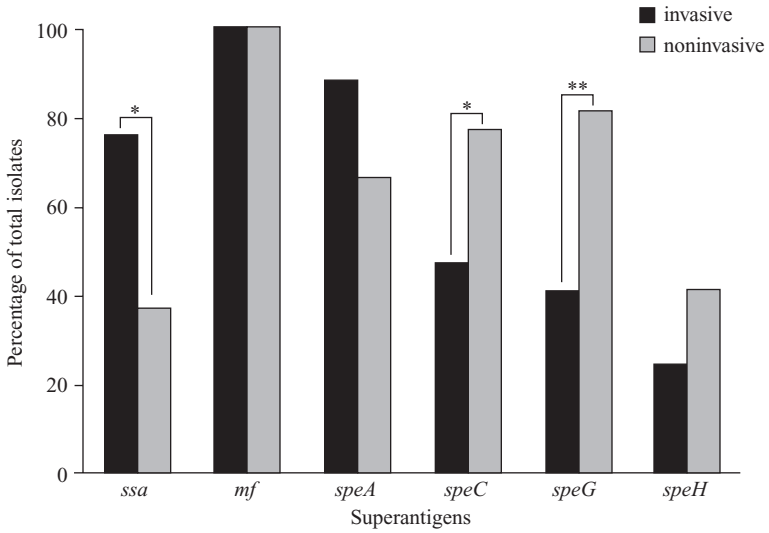

Fig. 3. Prevalence of superantigen genes among GAS isolates from invasive ($n = 17$) and non-invasive ($n = 299$) infections. * Fisher's exact text. $P < 0{\cdot}01$ and ** $P < 0{\cdot}001$.

than those from non-invasive patients (37%, $P < 0{\cdot}01$, Fig. 3). In addition, the occurrence of the *speA* gene in invasive isolates was 88%, while that in non-invasive isolates was 65·5%, ($P = 0{\cdot}064$) (Fig. 3). Since SPE-A and SSA are frequently found among various streptococcal superantigens, as has been revealed by amino acid sequencing [5], it is possible that these exotoxins are involved in the induction of toxic shock. However, our Western blot analysis showed that 47% of the invasive isolates expressed SPE-A, despite the finding that 88% possessed the *speA* gene. Nakashima et al. [24] reported that only 43% of the invasive isolates found produced SPE-A and 31% did not contain the *speA* gene. Therefore, when considering the amounts of toxins studied, there is a little evidence to establish whether SPE-A is closely connected with TSLS or invasive infection. Moreover, Hauser et al. [25] described other factors that are also likely to be important. Since not all of the strains from TSLS patients were found to contain the *speA* gene, further study by analysing more invasive isolates is required.

More recently, the complete genome sequence of a GAS M1 strain, SF370 (ATCC 700294), was

Table 2. *Superantigen genes among various* emm *genotype isolates*

		ssa			*speA*			*speC*			*speG*			*speH*		
emm type	No.	No.	%		No.	%		No.	%		No.	%		No.	%	
1	42	14	33		41	98	****	20	48	***	34	81		3	7	****
2	12	9	75	*	3	25	**	10	83		10	83		1	8	*
3	11	11	100	****	11	100	*	3	27	**	6	55		0	0	**
4	35	29	83	****	11	31	****	26	74		7	20	****	7	20	*
6	5	1	20		4	80		3	60		4	80		1	20	
11	11	2	18		9	82		9	82		11	100		2	18	
12	55	17	31		33	60		50	91	*	51	93	*	45	82	****
13	17	2	12		14	82		10	59		17	100		15	88	****
18	8	2	25		7	88		8	100		4	50		2	25	
28	53	17	32		37	70		42	79		48	91		24	45	
58	11	0	0	**	8	73		10	91		10	91		1	9	
75	7	1	14		5	71		6	86		7	100		5	71	
87	6	2	33		3	50		6	100		5	83		1	17	
89	8	0	0	*	5	63		5	63		7	88		2	25	
Others†	35	18	51		20	57		31	89		27	77		17	49	
Total	316	125	40		211	67		239	76		248	78		126	40	

* Fisher's $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$, **** $P < 0{\cdot}0001$.

† Small number of *emm* isolates (n < 5) were combined as 'Others'.

determined [5], and complete or partial sequences of four other bacteriophage genomes are known. In that study, the *speA/C/H/I* genes were shown to be associated with a phage gene [5], whereas the streptococcal mitogenic exotoxin Z, *speG* [6], and *mf* [21] appear to be chromosomally encoded. Since most isolates in our study harboured *speG* and all had *mf*, it was considered that SPE-G may not be a major virulence factor specific for invasive infection and chromosomally encoded superantigens might display less virulence.

Correlation of superantigen genes with *emm* genotypes

When the correlations between the *emm* genotype and superantigen profiles were analysed (Table 2), 41 of 42 genotype *emm1* isolates were found to possess the *speA* gene, and all of the genotype *emm3* isolates ($n = 11$) had the *speA* and *ssa* genes. The profiles of *speA* and *speC* genotypes in *emm1* isolates were *speA* (−) *speC* (+) ($n = 1$), *speA* (+) *speC* (−) ($n = 22$ with 3 invasive isolates) and *speA* (+) *speC* (+) ($n = 19$ with 1 invasive isolates), while those in *emm3* ($n = 11$) were *speA* (+) *speC* (−) ($n = 8$ with 5 invasive isolates) and *speA* (+) *speC* (+) ($n = 3$). Murase et al. [26] reported a comparison of superantigen profiles by PCR analysis in serotype M1/T1 and M3/T3 isolates during the periods 1981–8 and 1989–97. They showed that the *spe* genotype profiles in M1T1 isolates changed from *speA* (−) *speC* (−)/*speA* (−) *speC* (+) in the earlier period to *speA* (+) *speC* (−)/*speA* (+) *speC* (+) in the later period. In contrast, the profiles of almost all M3T3 isolates were *speA* (+) *speC* (+) during both periods and did not change. Our results were identical to their profiles in the later period, showing that genotype *emm1* strains with the *speA* gene have apparently increased in Japan as a result of the *speA*-associated phage gene.

Compared with the superantigen profiles of other *emm* isolates, isolates of genotype *emm2* (9 of 12) and *emm4* (29 of 35) possessed the *ssa* gene at ratios of 75% and 83%, respectively ($P < 0{\cdot}01$). Among a total of 55 genotype *emm12* isolates, 50, 51, and 45 isolates possessed *speC* ($P < 0{\cdot}01$), *speG* ($P < 0{\cdot}01$), and *speH* ($P < 0{\cdot}0001$), respectively. Further, 15 of 17 genotype *emm13* isolates harboured *speH* ($P < 0{\cdot}01$). It was noteworthy that a significant number of genotype *emm4* isolates did not have the *speG* gene (Table 2), whereas many others possessed it. These results suggest that the distribution of *emm* genotypes is related to superantigens, and are the first to show the profiles of *speG* and *speH*. However, *speH* has not been characterized precisely, and more information is needed to clarify the association between the clinical features and pathogenic roles of SPE-H.

Ferretti et al. [5] also showed that the location of virulence-associated genes near the integration site

of each complete phage and the ubiquitous presence of phages in GAS assure the possibility of horizontal gene transfers by these virulence determinants, which would play an important role in increasing the pathogenic potential of the organism as well as in its overall evolution. However, Sriskandan et al. [22] considered that the chromosomally encoded virulence factors must account for the lethality of invasive organisms. Therefore, it seems important to examine the role of phage-associated genes or chromosomally encoded genes as possible virulence factors in invasive GAS infections.

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