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Epidemiological Study of Erythromycin-Resistant *Streptococcus pyogenes* From Korea and Japan by *emm* Genotyping and Multilocus Sequence Typing

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Background: We determined the epidemiological characteristics of erythromycin (EM)-resistant *Streptococcus pyogenes* (group A streptococci, GAS) strains isolated from Korea and Japan, using *emm* genotyping and multilocus sequence typing (MLST).

Methods: Clinical isolates of GAS had been collected from 1992 to 2012 in Korea and from 2004 to 2009 in Japan. EM resistance was determined by the microdilution method, and resistance genotypes were assessed by PCR. The *emm* genotyping and MLST were performed by DNA sequencing.

Results: The *emm* genotypes and sequence types (STs) were concordant in 143 (85.1%) of 168 EM-resistant GAS strains from Korea. ST36/*emm12* (35.1%), ST52/*emm28* (22.6%), and ST49/*emm75* (16.1%) were the most common types. Most of the ST36 (93.9%) and ST52 (95.8%) strains harbored *erm*(B), whereas strains ST49, ST42, and ST15 contained *mef*(A). The concordance between *emm* genotypes and STs was 41 (93.2%) among 44 EM-resistant GAS strains from Japan. ST36/*emm12* (34.1%), ST49/*emm75* (18.2%), and ST28/*emm1* (15.9%) were the major types. ST36 isolates harbored either *erm*(B) (56.3%) or *mef*(A) (37.5%), whereas isolates ST28, ST49, and ST38 carried only *mef*(A). The proportion of *erm*(B) and *mef*(A) was 66.1% and 33.3% in Korea and 22.7% and 68.2% in Japan, respectively.

Conclusions: The common STs in Korea and Japan were ST36 and ST49, whereas ST52 was present only in Korea and ST28 only in Japan. Genotype *erm*(B) was predominant in Korea, whereas *mef*(A) was frequent in Japan. There were differences between Korea and Japan regarding the frequencies of *emm* genotypes, STs, and EM resistance genes among the EM-resistant GAS.

Key Words: *Streptococcus pyogenes*, Genotypes, Sequence types, Erythromycin, Resistance

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INTRODUCTION

Streptococcus pyogenes (group A streptococci, GAS) is the most common causative pathogen of acute pharyngitis, with an especially high incidence among preschool children. This pathogen can induce several immunological sequelae, such as rheumatic fever, rheumatic heart diseases, and acute poststreptococcal glomerulonephritis, although acute GAS pharyngitis it-

self is a mild illness [1]. In addition, severe cases of necrotizing fasciitis or toxic shock-like syndrome induced by GAS (so-called invasive GAS infections) have been described among adults [2].

β-Lactam antimicrobial agents, including penicillin, are the first line therapy to treat streptococcal pharyngitis, but erythromycin (EM) or other macrolide agents can be administered to patients who are allergic to the β-lactams. However, the resistant rates of GAS to macrolides, including EM, have increased in Korea [3], where the resistance rate to EM among the 125 strains recovered from throat cultures during 2001-2002 was 44.8% [4]. Interestingly, the EM resistance rate of GAS isolated from children with acute pharyngitis decreased significantly in 2009 (4.6%) (P=0.0000) [5]. We observed a remarkable change in the distribution of *emm* genotypes during this period in Korea [5].

Multilocus sequence typing (MLST) is a method that can be used to investigate the molecular epidemiology and population genetic structure of pathogenic bacteria. Enright *et al*. [6] developed an MLST scheme for GAS, and the nucleotide sequences of internal fragments of seven selected housekeeping loci (glucose kinase, glutamine transporter, glutamate racemase, DNA mismatch repair protein, transketolase, xanthine phosphoribosyltransferase, and acetyl-CoA acetyltransferase) were established to identify combinations of housekeeping alleles (allelic profiles). Stable associations between the *emm* genotypes and sequence types (STs) were documented by comparing GAS strains obtained decades apart and/or from different regions. However, MLST has not been applied to determine the endemic clones among EM-resistant strains in Korea. Wajima *et al*. [7] recently reported an association of macrolide resistance with molecular typing results (STs and *emm* genotypes) in 363 GAS isolates from pharyngotonsillitis in Japan. However, epidemiological data from comparison of GAS isolates between Korea and Japan are still sparse.

The aim of this study was to compare the molecular epidemiological characteristics of EM-resistant GAS isolates from 1992 to 2012 in Korea and from 2004 to 2009 in Japan, using the data of *emm* genotypes, STs, and EM resistance genes.

METHODS

1. Sample collection and bacterial isolates

Clinical specimens (mainly throat swabs) were taken from patients who had visited either clinics or hospitals in Jinju, Gyeongnam, Korea, from 1992 to 2012. We also collected clinical specimens (mainly throat swabs) from individuals who visited either clinics or hospitals in Saitama prefecture or Miyagi prefecture, Japan, from 2004 to 2009. Each specimen was inoculated on a 5% sheep blood agar plate and kept in a 35°C incubator overnight. Gray-white colonies exhibiting β-hemolysis were subjected to latex agglutination testing with Lancefield group A-specific antisera (Seroiden Strepto kit; Eiken Chemical Co., Ltd., Tokyo, Japan) [4, 5]. We collected 168 EM-resistant GAS isolates from 1992 to 2012 in Korea. The total number of isolates per year was 2, 12, 85, 32, 5, 14, and 18 in 1992, 1995, 2002, 2004, 2006, 2009, and 2012, respectively. These isolates were derived predominantly from throat samples (n=154) as well as from pus (n=7), sterile specimens (i.e., blood, joint fluid, and tissue; n=5), sputum (n=1), and urine (n=1). A total of 44 EM-resistant strains were collected from 2004 to 2009 in Japan. The total number of isolates per year was 4, 2, 5, 2, 27, and 4 in 2004, 2005, 2006, 2007, 2008, and 2009, respectively. These strains were isolated from throat samples (n=21) or nasal specimens (n=23). Pediatric subjects made up the majority of the registered-patient populations in both Korea (n=153, 91.1%) and Japan (n=34, 77.3%). All of the isolates identified as GAS (one isolate per patient) were stored at -70°C until being processed for further evaluation.

2. Antimicrobial susceptibility testing

Minimal inhibitory concentrations (MICs) of the antimicrobial agents EM, azithromycin, clindamycin (CLI), levofloxacin, tetracycline, and penicillin G were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines for β-hemolytic streptococci [8]. We used Eiken Dry Plates (Eiken Chemical Co., Ltd., Tokyo, Japan) and Mueller-Hinton broth supplemented with 5% lysed horse blood to measure the MICs of these agents [9]. The EM-resistant isolates were selected through this method. Additionally, the CLI-resistant strains were chosen since the phenotypic expression of EM resistance is divided into constitutive resistance, inducible resistance, or M phenotype. To reduce interlaboratory bias, the antimicrobial susceptibility testing of all Korean and Japanese isolates was performed at Laboratory of Infectious Diseases, Kitasato Institute for Life Sciences & Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan.

3. Detection of EM resistance genes

All EM-resistant GAS strains were screened for causative resistance determinants [4]. Briefly, the *erm*(B), *erm*(A), and *mef*(A) genes were detected by using PCR-based amplification with specific primer sets [10] and extracted bacterial DNA templates. The PCR products were examined by 1.5% agarose gel electro-

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phoresis in TBE buffer (Tris/borate/EDTA, pH 8.0).

We performed *emm* genotyping on all EM-resistant GAS isolates. Briefly, the *emm* gene was amplified by using PCR techniques with a specific primer set [11] and the bacterial DNA template. The amplified products were purified by using an AccuPrep Purification Kit (Bioneer, Daejeon, Korea), and their nucleotide sequences were determined. All *emm* genotyping was based on the Centers for Disease Control and Prevention database (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp).

4. Multilocus sequence typing

This procedure was performed by sequencing seven housekeeping genes (*gki*, *gtr*, *murI*, *mutS*, *recP*, *xpt*, and *yqiL*) according to the MLST website for GAS (http://spyogenes.mlst.net/) [6]. The determined sequences in the seven genes were entered into the website input field after trimming the 5′- and 3′-ends, and thereafter, the corresponding allelic profile and ST for each isolate was obtained from the website database.

5. Clustering analysis by eBURST

Clustering analysis to evaluate relationships among the STs was performed with the online-available eBURST software (http://spyogenes.mlst.net/eburst/) as previously described [12]. EM resistance gene-defined subsets of the isolates were separately analyzed by eBURST. Clonal complexes (CCs) consisting of single-locus variants and double-locus variants were identified with a user-defined setting of >5 shared housekeeping alleles among the seven sequenced genes. Identification of the founder ST for a CC was ascertained by bootstrap analysis using 1,000 replicates [12].

6. Statistical analysis

Distributions of the EM-resistant STs at different time points were compared by using the chi-square test. A *P* value of <0.05 indicated statistical significance.

RESULTS

1. Antimicrobial resistance

The MIC_{50}/MIC_{90} values were 16/>512 for EM and 0.5/>128 for CLI against the 168 Korean isolates, and >16/>16 for EM and 0.12/>16 for CLI against the 44 Japanese isolates. CLI-resistant strains accounted for 66.1% of the total isolates from Korea and 25.0% of those from Japan. The resistance rates against azithromycin, levofloxacin, tetracycline, and penicillin G, respectively, were 100%, 2.4%, 67.3%, and 0% for the Korean isolates and 95.5%, 0%, 29.5%, and 0% for the Japanese isolates.

2. EM resistance genes

Among the 168 EM-resistant strains from Korea, 66% harbored *erm*(B) and the remaining 33.3% had *mef*(A). There were no isolates with *erm*(A). On the other hand, among the 44 isolates from Japan, 22.7% harbored *erm*(B), 9.1% had *erm*(A), and 68.2% contained *mef*(A).

3. *emm* genotyping

Fourteen different *emm* genotypes were identified in the Korean isolates: the major types were *emm12* (n=60, 35.7%), *emm28* (n=38, 22.6%), *emm75* (n=28, 16.7%), and *emm18* (n=14, 8.3%) (Table 1). The *emm12* or *emm28* strains had *erm*(B) except for three isolates, whereas the *emm75* or *emm18* strains harbored *mef*(A) except for two isolates. On the other hand, nine different *emm* genotypes were confirmed in the Japanese isolates: the major types were *emm12* (n=17, 38.6%), *emm1* (n=8, 18.2%), *emm75* (n=8, 18.2%), and *emm4* (n=4, 9.1%). The *emm12* strains had *erm*(B) (n=8) or *mef*(A) (n=9), whereas the *emm1* (n=7), *emm75* (n=8), and *emm4* (n=3) strains harbored only *mef*(A).

4. Multilocus sequence typing

Results of the discrepancy between STs and *emm* major genotypes, and the comparison between STs/*emm* genotypes and EM resistance genotypes are presented in Table 1. Nine different STs were identified for the 168 EM-resistant isolates from Korea, although we could not determine STs for four isolates. The major STs were ST36 (n=66, 39.3%), ST52 (n=48, 28.5%), and ST49 (n=29, 17.3%). The concordance between *emm* genotypes and STs was 85.1% (143 of 168). Most of the ST36 and ST52 strains harbored *erm*(B) (93.9% and 95.8%, respectively), whereas the ST15, ST38, ST42, ST49, ST55, and ST75 strains carried only the *mef*(A) resistance gene. On the other hand, 10 different STs were observed in the isolates from Japan. The major STs were ST36 (n=16, 36.4%), ST28 (n=9, 20.5%), and ST49 (n=8, 18.2%). The concordance between *emm* genotypes and STs was 93.2% (41 of 44). The ST36 isolates harbored either *mef*(A) (n=6), *erm*(A) (n=1), or *erm*(B) (n=9), whereas the ST15, ST28, ST38, ST39, ST49, and ST551 isolates carried only *mef*(A). ST63 (n=2) and ST176 (n=1) contained only *erm*(A).

5. Clustering analysis by eBURST

Table 2 indicates the genetic relatedness among STs comprising

Table 1. Relationships between sequence types, *emm* genotypes, and antimicrobial resistance and macrolide resistance genes among *Streptococcus pyogenes* isolates from Korea and Japan

Country	Sequence types (%)	*emm* genotypes (N)	N of antimicrobial resistance		N of antimicrobial resistance genes		
			Erythromycin	Clindamycin	*mef*(A)	*erm*(A)	*erm*(B)
Korea	36 (39.3)	12 (59), 18 (3), 3 (1), 75 (1), ND (2)	66	62	3		62
	52 (28.5)	28 (38), 77 (7), 18 (1), 58 (1), 88 (1)	48	44	2		46
	49 (17.3)	75 (27), 18 (2)	29	1	29		
	42 (4.8)	18 (6), 3 (1), 12 (1)	8	1	8		
	15 (3.5)	3 (6)	6		6		
	55 (1.8)	2 (3)	3		3		
	75 (1.2)	9 (2)	2		2		
	38 (0.6)	4 (1)	1		1		
	101 (0.6)	89 (1)	1	1			1
	ND (2.4)	18 (2), 86 (1), 123 (1)	4	2	2		2
	Total N (%)		168 (100)	111 (66.1)	56 (33.3)		111 (66.1)
Japan	36 (36.4)	12 (15), 28 (1)	16	9	6	1	9
	28 (20.5)	1 (7), 12 (1), 28 (1)	9		9		
	49 (18.2)	75 (8)	8	1	8		
	38 (9.1)	4 (3), 1 (1)	4		4		
	63 (4.5)	77 (2)	2			2	
	15 (2.3)	3 (1)	1		1		
	39 (2.3)	4 (1)	1		1		
	176 (2.3)	58 (1)	1			1	
	382 (2.3)	6 (1)	1	1			1
	551 (2.3)	12 (1)	1		1		
	Total N (%)		44 (100)	11 (25.0)	30 (68.2)	4 (9.1)	10 (22.7)

Blank cells represent zero.
Abbreviation: ND, not determined.

Table 2. Clustering of sequence types comprising each erythromycin resistance gene-defined group of *Streptococcus pyogenes* isolates from Korea and Japan

Country	EM resistance gene	N of strains	STs*	N of CCs†	N of singleton clones
Korea	*mef*(A)	56	ST15, ST36, ST38, ST42, ST49, ST52, ST55, ST75	0	8
	erm(B)	111	ST36, ST52, ST101	0	3
Japan	*mef*(A)	30	ST15, ST28, ST36, ST38, ST39, ST49, ST551	2	3
	erm(A)	4	ST36, ST63, ST176	0	3
	erm(B)	10	ST36, ST382	0	2

*There were four isolates without determined STs in Korea; †CC is defined as STs sharing more than five of seven housekeeping alleles.
Abbreviations: EM, erythromycin; STs, sequence types; CCs, clonal complexes.

each EM resistance gene-defined group of the isolates from Korea and Japan. In the Korean isolates, all of the corresponding ST groups harboring *mef*(A) or *erm*(B) (total ST number, 8 and 3, respectively) were singleton clones. Genetic relatedness was also observed among the STs comprising each EM resistance gene-defined group of the Japanese isolates. In this case, whereas all the corresponding ST groups harboring *erm*(A) or *erm*(B) (total ST number, 3 and 2, respectively) were singleton clones, there were two CCs (ST36 and ST551; ST38 and ST39) and three singleton clones among the ST group with *mef*(A).

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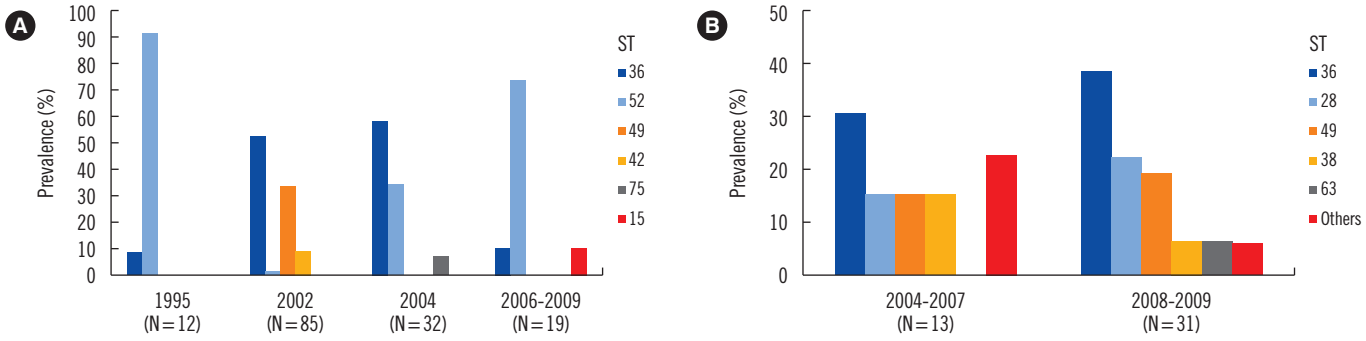

Fig. 1. Prevalence of sequence types (STs) by different periods in Korea (A) and in Japan (B).

6. Clonal change of EM-resistant strains

The prevalence rates of STs of the EM-resistant isolates from Korea and Japan are shown in Fig. 1A and B, respectively. In 2002, ST36 was predominant (46.8%) in Korea, whereas ST52 was rare (1.1%). However, the prevalence of ST36 declined (7.1%) and that of ST52 increased remarkably (71.4%) during 2006-2009. ST49 was observed only in 2002. The distribution of the STs between 2002 and 2006-2009 in Korea was significantly different (P=0.0000). In Japan, ST36 was predominant in both periods of 2004-2007 and 2008-2009.

DISCUSSION

An epidemiological study and molecular characterization of EM-resistant GAS strains (n=127) in comparison with randomly selected EM-susceptible isolates (n=128) was reported in Taiwan over three different research periods (1998-2000, 2002-2004, and 2006-2010) [13]. Interestingly, the EM-resistant strains of ST36/*emm12* type with the constitutive resistance phenotype were most prevalent before 2004. We selected only EM-resistant strains in this study. It may be necessary to compare the molecular characteristics of EM-resistant strains with EM-susceptible strains in our countries. As the EM resistance rate was low in Japan [14], only a small number of the isolates were included for our analysis.

Furthermore, a molecular analysis of GAS isolates (n=185) from children with pharyngitis has been performed in China, and the *emm12* strains were found to belong to ST36, whereas the *emm1* isolates were designated to ST28 [15]. These findings were consistent with our observations. Clonal impact by the EM-resistant strains having ST36/*emm12* genotype appeared to occur in Taiwan as well as in Korea before 2004. There was a sudden increase in the frequency of ST36/*emm12* and it was strongly associated with EM resistance in 2002 in Korea [4, 5]. In the analysis of periodical change of STs/*emm* genotypes in Japan, ST36/*emm12* was consistently predominant throughout the study period.

Association of macrolide resistance with molecular typing data (STs and *emm* genotypes) has been documented among macrolide-susceptible or macrolide-resistant GAS isolates from noninvasive infections in 2012 in Japan [7]. Much as in the present study, these authors found that ST28/*emm1* (24.0%) and ST36/*emm12* (23.4%) were the most prevalent types. This strategy has also been adopted using the strains (n=283) from invasive infections from 2010 to 2012 in Japan [16]. Our molecular typing results with the isolates collected from 2004 to 2009 seem to be useful to sequentially estimate the epidemiological data regarding the EM-resistant isolates in the areas of Saitama and Miyagi prefectures, Japan. There was a difference in ST distribution between Korea and Japan in this study. For example, the second most common sequence type, ST52, which comprised 28.5% of the isolates from Korea, was not observed in the Japanese isolates. Likewise, the second most common sequence type, ST28, which comprised 20.5% of the Japanese isolates, was not identified in the Korean isolates. Although there is no clear evidence of such, there might have been an outbreak of EM-resistant strains in 2002 in Korea and in 2008 in Japan, because the numbers of EM-resistant strains increased sharply in these periods. Moreover, the frequency of EM resistance genes was different between Korea and Japan. The *erm*(B) gene suggestive of constitutive resistance was predominant in Korea, whereas the *mef*(A) gene indicative of M phenotype was prevalent in Japan. Interestingly, almost all the strains analyzed (100% in Korea and 95.5% in Japan) were resistant to azithromycin (15-membered ring), suggesting EM (14-membered ring) as the representative drug for susceptibility of the macrolides [8].

In the analysis of CCs according to EM resistance genes,

there were no CCs in the Korean isolates. The CCs were observed only among the *mef*(A)-harboring isolates from Japan. This finding suggests there might be independent genetic events contributing to the spread of EM-resistant strains in both countries. In the future, it would be useful to monitor the genetic relatedness among STs comprising each EM resistance gene-defined group of the isolates.

In conclusion, the common STs in both Korea and Japan were ST36 and ST49, whereas ST52 was present only in Korea and ST28 only in Japan. The *erm*(B) genotype was predominant in Korea, whereas *mef*(A) was frequent in Japan. There were considerable differences between Korea and Japan in the frequencies of *emm* genotypes, STs, and EM resistance genes for the EM-resistant GAS isolates.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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