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Original Article

Highly prevalent *emmSTG840.0* and *emmSTC839.0* types of erythromycin non-susceptible group G *Streptococcus* isolated from bacteremia in southern Taiwan

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Abstract *Background/Purpose:* Group G *Streptococcus* (GGS) infections in human have increased. Treatment relied on antibiotic therapy, including erythromycin. However, information regarding the dominant strains and erythromycin susceptibility in GGS bacteremia is limited.
Methods: A total of 134 GGS were isolated from patients with bacteremia in a university hospital of southern Taiwan during 1993–2010. The erythromycin susceptibility was determined by disc diffusion and agar dilution assays. The bacterial species was determined by MALDI-TOF. The presence of erythromycin-resistant genes and *emm* types were determined by polymerase chain reaction and sequence. The clonal spreading was analyzed by pulsed-field gel electrophoresis with *Sma*I or *Sgr*AI digestion.
Results: The annual erythromycin non-susceptible rate varied, with an average of 40.3%. All erythromycin non-susceptible strains belonged to the *Streptococcus dysgalactiae*. No

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erythromycin non-susceptible strains belong to the *anginosus* group. The most prevalent erythromycin-resistant gene was *mefA* (57.4%), followed by *ermB* (37%), and *ermA* (3.7%). The N terminal hyper variable region of *emm* was sequenced to determine the *emm* type, and only *S. dysgalactiae* had the *emm* gene. The most prevalent *emm* types were *emmSTG840.0* (17.2%), *emmSTG485.0* (10.4%), and *emmSTC839.0* (9.0%). 73% and 47% of the strains with only *mefA* and *ermB* belonged to *emmSTG840.0* and *emmSTC839.0* types, respectively. Pulsed-field gel electrophoresis showed that different clones of *emmSTG840.0* and *emmSTC839.0* strains were spread in this region during the 18 years of surveillance.

Conclusion: Our data indicate that there were dominant *emm* types with erythromycin non-susceptibility in *S. dysgalactiae* isolated from bacteremia in Taiwan, and thus constant surveillance is warranted.

Introduction

Group G *Streptococcus* (GGS) is commensals and pathogens in domestic animals. Recently, increased GGS infections in humans have been reported, and disease outcomes are diverse, including pharyngitis, cellulitis, meningitis, endocarditis, and sepsis,[1–4] which are similar to group A *Streptococcus* (GAS).

There are many species of GGS, including *Streptococcus dysgalactiae*, *Streptococcus anginosus*, and *Streptococcus constellatus*.[5] *S. dysgalactiae* is the major source of GGS infection in human. *S. anginosus* is commonly isolated from urogenital and gastrointestinal sources, while *S. constellatus* is often isolated from the respiratory tract.[6] Since the classification of *S. anginosus* and *S. constellatus* is complex, these two species were classified as *anginosus* group.[6] Penicillin G is the first choice for GGS-infected patients, while macrolides, including erythromycin (EM), are the alternative choice for type I allergic patients.[7] Several studies found that the EM non-susceptible rates of GGS were varied from 0 to 42.5%.[8,9] In the strains isolated from bacteremia, the EM non-susceptible rate was 7%.[10] The mechanisms leading to EM resistance include modification of 23s rRNA and presence of an efflux pump. ErmA (previously nominated as ErmTR) and ErmB modify 23s rRNA, leading to inducible and constitutive macrolide–lincosamide–streptogramin phenotypes (iMLS or cMLS). ErmC can also modify 23s rRNA, but it can generate cMLS or iMLS. MefA is an efflux pump, which can pump out EM and lead to resistance to macrolides (M phenotype).[11–13]

Since the genome of *S. dysgalactiae* is similar to GAS, the *emm* typing of GAS can also be applied to *S. dysgalactiae*.[2,4,14] Currently, more than 60 different *emm* types were found in *S. dysgalactiae*.[7] In this study, a total of 134 GGS isolated from blood were collected in Southern Taiwan during 1993–2010. The species, *emm* type, and antibiotic resistance were determined to uncover the epidemiological trends of GGS in Taiwan.

Materials and methods

Strain collection and identification

GGS were collected in the Department of Pathology, National Cheng Kung University Hospital in southern Taiwan. One hundred and thirty-four GGS isolates were collected from blood during 1993–2010. GGS was identified by the latex agglutination method (Oxoid, basingstoke, United Kingdom). The bacterial species was identified by a MALDI Biotyper (Bruker, MA, USA).[15,16] *S. anginosus* and *S. constellatus* were classified as the *anginosus* group.

Susceptibility testing

Susceptibilities to EM and clindamycin were identified by disc diffusion assay according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI).[17] The EM minimal inhibition concentration (MIC) of the strains with EM resistance and intermediate based on disc diffusion assay was further determined by agar dilution assay according to CLSI guideline. The MIC interpretive criteria were also based on CLSI guideline. If the interpretations from disc diffusion and MIC were different, we chose MIC to interpret EM susceptibility. Only the strains with EM intermediate and resistance were defined as EM non-susceptible stains. The cMLS, iMLS, and M phenotypes were determined by double disc diffusion assay. Briefly, EM non-susceptible strains with McFarland 0.5 turbidity were plated on Muller-Hinton agar supplemented with sheep blood (5% v/v). The 15 mg EM disk and 2 mg clindamycin disk were placed 12 mm apart, and the plates were inoculated in 5% CO_2 condition at 37 °C for 20–24 h. The cMLS phenotype was defined as resistance to EM and clindamycin. The M phenotype was defined as resistance to EM. The iMLS phenotype was determined by the feature that flattening inhibition zone adjacent to the EM disk and should be considered as clindamycin resistance.

DNA extraction and gene detection

Genomic DNA was extracted by FavorPrep™ tissue genomic DNA extraction mini kit according to the instruction manual (Favorgen, Ping-Tung, Taiwan). EM-resistant genes, including *mefA*, *ermC*, *ermB*, and *ermA*, were detected by polymerase chain reaction (PCR) according to a previous description.[12,18,19] Primer sequences are listed in Table 1.

Table 1 The primer sequences used in this study.

Target genes	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
ermB	gaaaaggtactcaaccaaata agtaacggtacttaaattgtttac	639	19
mefA	agtatcattaatcactagtgc ttcttctggtactaaaagtgg	348	19
ermA	atagaaattgggtcaggaaaagg ttgatttttagtaaaaag	530	19
ermC	tcaaaacataatatagataaa gctaatattgtttaaatcgtcaat	642	12,18
emm	tatt(c/g)gcttagaaaattaa gcaagttcttcagcttgttt	Varied	33
emm-seq2	tattcgcttagaaaattaaaaacagg		33

Determination of *emm* type

PCR amplification, primers, and sequencing of *emm* genes were performed according to the CDC guideline (http://www.cdc.gov/streplab/protocol-emm-type.html). Briefly, the *emm* gene was amplified by the primers emm-1 and emm-2, and sequenced by the primer emm-Seq2 (primer sequences are listed in Table 1). The *emm* types of each strain were determined in the *emm* type-specific CDC database (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp). The new *emm* type (*emmSTG20.0*) was registered and nominated in the *emm* type-specific CDC database.

Pulsed-field gel electrophoresis (PFGE)

The genomic DNA of GGS was extracted and digested by *Sma*I or *Sgr*AI according to a previous description.[20] PFGE was performed on a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA) according to the instruction manual. PFGE fragment patterns were compared by using the unweighted pair group method using average linkages (UPGMA) based on the Dice coefficient with 1.0% optimization and 1.5% band tolerance in BioNumerics version 6.5 (Applied Maths NV). The strains sharing more than 80% of similarity were considered the same clone.

Statistical analysis

To correlate EM non-susceptibility and species, Fisher's exact test was used. To estimate the significance of observed changes of annual prevalence rates of the *ermB* and *mefA* genes, and annual non-susceptible rate to EM, Spearman correlation was used. To estimate the association between EM susceptibility and *emm* type, the multinomial logistic regression was used. Briefly, since *emmSTG840.0*, *emmSTG485.0*, *emmSTC839.0*, *emmSTG652.0*, and *emmSTG6.1* comprised 54.6% of all isolates (Table 3), only these five *emm* types were chosen to estimate the associations. The other *emm* types were grouped into "the other *emm* type" (Table 3). The statistical analysis was interpreted by comparing the top five *emm* types to "the other *emm* type". A p value less than 0.05 was considered as statistical significance. All of the statistical analyses were performed in SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Erythromycin susceptibility in group G *Streptococcus*

A total 134 isolates were collected from 1993 to 2010 (Fig. 1). Among them, 119 (88.8%) strains were belonged to *S. dysgalactiae*, and the other 15 strains (11.2%) were belonged to *anginosus* group (Fig. 1). Fifty-four strains (40.3%) were EM non-susceptible and belonged to the *S. dysgalactiae*, while there were no EM non-susceptible strains in the *anginosus* group. EM non-susceptibility was significantly associated with the *S. dysgalactiae* ($p < 0.01$). The annual EM non-susceptible rates varied each year. Overall, there was no significant change of the annual EM non-susceptible rate during 1993–2010 (Fig. 1, $r = -0.12$. $p = 0.62$).

The distributions of erythromycin-resistant genes and their phenotypes

Among 54 EM non-susceptible strains, 19, 30, and 2 strains had only *ermB*, *mefA*, and *ermA* genes, respectively (Table 2). *ermC* gene was not found. One EM non-susceptible strain had both of *ermB* and *mefA* gene, but its EM MIC was 0.5 mg/L, which was interpreted as EM intermediate (Table 2). All of the strains with only *ermB* or only *mefA* had EM MIC higher than 256 mg/L or 1–16 mg/L, respectively (Table 2). The EM MIC of the strains with *ermA* was 32 mg/L (Table 2). To confirm the resistant activities of *ermB*, *mefA*, and *ermA* genes, the correlation between phenotype and

Table 2 Phenotype and genotype correlations in 54 erythromycin non-susceptible group G *Streptococcus*.

Phenotype (No. of isolates)	Genotype	No. of strains (%)	Erythromycin MIC_{50} (Range) (mg/L)
cMLS (26)	*ermB*+	19 (35)	>256 (256–>256)
	mefA+	5 (9)	16 (1–16)
	ermB+ *mefA*+	1 (2)	0.5
	ermA− *ermB*− *ermC*− *mefA*−	1 (2)	16
M phenotype (26)	*mefA*+	25 (46)	16 (1–16)
	ermA− *ermB*− *ermC*− *mefA*−	1 (2)	2
iMLS (2)	*ermA*+	2 (4)	32 (32–32)

Table 3 The prevalence of species, *emm* type, and erythromycin (EM) susceptibility in group G *Streptococcus*.

Species and *emm* type	No. of strains (%)			No. of EM-non susceptible strains (%)				
	EM non-susceptible	EM susceptible	Total	*ermB*	*mefA*	*ermA*	*ermB* and *mefA*	Unknown
anginosus group								
Nontypable	0 (0)	15 (100)	15 (100)	–	–	–	–	–
S. dysgalactiae								
STG840.0	22 (96)	1 (4)	23 (100)	0 (0)	22 (73)	0 (0)	0 (0)	0 (0)
STG485.0	5 (36)	9 (64)	14 (100)	1 (5)	3 (10)	0 (0)	1 (100)	0 (0)
STC839.0	9 (75)	3 (25)	12 (100)	9 (47)	0 (0)	0 (0)	0 (0)	0 (0)
STG652.0	0 (0)	9 (100)	9 (100)	–	–	–	–	–
STG6.1	1 (14)	6 (86)	7 (100)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
STG166B.0[a]	2 (33)	4 (66)	6 (100)	0 (0)	2 (7)	0 (0)	0 (0)	0 (0)
STG10.0[a]	2 (40)	3 (60)	5 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
STG245.0[a]	3 (60)	2 (40)	5 (100)	2 (11)	1 (3)	0 (0)	0 (0)	0 (0)
STC6979.0[a]	3 (75)	1 (25)	4 (100)	3 (16)	0 (0)	0 (0)	0 (0)	0 (0)
STC74A.0[a]	0 (0)	4 (100)	4 (100)	–	–	–	–	–
STG480.0[a]	0 (0)	4 (100)	4 (100)	–	–	–	–	–
STG652.1[a]	0 (0)	4 (100)	4 (100)	–	–	–	–	–
STG120.0[a]	1 (33)	2 (67)	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	1 (50)
STG351.1[a]	0 (0)	3 (100)	3 (100)	–	–	–	–	–
STG6.0[a]	2 (67)	1 (33)	3 (100)	0 (0)	1 (3)	0 (0)	0 (0)	1 (50)
STG5420.0[a]	0 (0)	2 (100)	2 (100)	–	–	–	–	–
STGLP1.0[a]	0 (0)	2 (100)	2 (100)	–	–	–	–	–
STC1400.0[a]	0 (0)	1 (100)	1 (100)	–	–	–	–	–
STC5345.0[a]	1 (100)	0 (0)	1 (100)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
STG211.1[a]	0 (0)	1 (100)	1 (100)	–	–	–	–	–
STG4222.0[a]	0 (0)	1 (100)	1 (100)	–	–	–	–	–
STG4831.0[a]	0 (0)	1 (100)	1 (100)	–	–	–	–	–
STG62647.0[a]	0 (0)	1 (100)	1 (100)	–	–	–	–	–
STG653.0[a]	1 (100)	0 (0)	1 (100)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
STG20.0[a]	1 (100)	0 (0)	1 (100)	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)
Nontypable[a]	1 (100)	0 (0)	1 (100)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)
Total (*S. dysgalactiae*)	54 (45)	65 (55)	119 (100)	19 (100)	30 (100)	2 (100)	1 (100)	2 (100)

[a] To perform statistical analysis, these *emm* types were grouped as "the other *emm* type" (see the descriptions in Materials and methods).

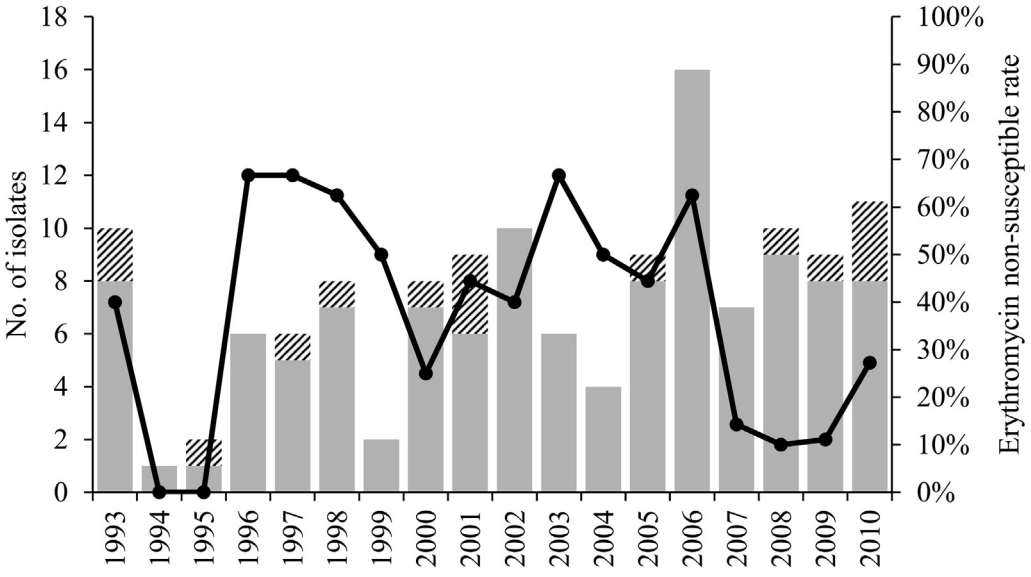

Figure 1. Changes in the percentage of erythromycin (EM) non-susceptible GGS isolates during 1993 to 2010. A total of 134 GGS were collected. The gray and slash bars indicate the number of isolates belonging to *S. dysgalactiae* and *anginosus* group, respectively. The black line indicates the EM non-susceptible rate.

genotype of macrolide resistance was analyzed. All *ermB*- and *ermA*-containing strains had cMLS and iMLS phenotypes, respectively (Table 2). In the strains with M phenotype, twenty-five strains had *mefA* (Table 2). However, six strains with *mefA* harbored cMLS. Two strains without *ermA*, *ermB*, *ermC*, or *mefA* had cMLS and M phenotypes, and their EM MIC was 16 and 2 mg/L, respectively, suggesting unknown resistant mechanisms existed in GGS.

When comparing the prevalence of EM-resistant genes and isolation year, the annual prevalence of a single *ermB* gene was significantly increased ($r = 0.70$, $p = 0.0013$), and the annual prevalence of a single *mefA* gene was significantly decreased ($r = -0.47$. $p = 0.0466$). The *ermA* gene was only present in 2010. Two EM non-susceptible strains without *ermA*, *ermB*, *ermC*, or *mefA* genes were present in 2000 and 2006.

The prevalence of *emm* types and their association to erythromycin susceptibility

Among 134 GGS isolates, the *emm* gene was not detected in the *anginosus* group (Table 3). In the *S. dysgalactiae*, other than one strain lacking an *emm* gene, 25 different *emm* types were found (Table 3). One new *emm* type (*emmSTG20.0*) was found, which shared 83% identity to *emmSTG840.3*. The *emmSTG840.0*, *emmSTG485.0*, *emmSTC839.0*, *emmSTG653.0*, and *emmSTG6.1* types were the top five most prevalent *emm* types. The *emmSTG840.0* and *emmSTC839.0* were significantly associated with EM non-susceptibility ($p = 0.0003$ and 0.001, respectively).

The association between *emm* types and EM-resistant genes was further compared. The results showed that 73% of the strains with only *mefA* belonged to *emmSTG840.0* ($p < 0.0001$, Table 3), while 47% of the strains with only *ermB* belonged to *emmSTC839.0* ($p < 0.0001$, Table 3). PFGE was performed in *emmSTG840.0* and *emmSTC839.0* strains to determine their PFGE patterns. In *emmSTG840.0* strains, two different PFGE patterns were found (Fig. 2A). In twelve *emmSTC839.0* strains, ten strains were resistant to *Sma*I digestion, and two strains had the PFGE pattern B, which was also present in *emmSTG840.0* strains (Fig. 2A). When genomic DNA of *emmSTC839.0* strains was further digested by *Sgr*AI, twelve *emmSTC839.0* strains can be further divided into five different patterns (Fig. 2B).

The association between isolation year and strain distribution was further investigated. Among 14 *emmSTG840.0* strains with PFGE pattern A, 13 strains were isolated before 2003, while all of the *emmSTG840.0* strains having PFGE pattern B were found after 2004 (Fig. 2A). The distribution of PFGE patterns A and B in *emmSTG840.0* strains was significantly associated with isolation year ($p < 0.001$), suggesting that two clones had different temporal distribution. However, in *emmSTC839.0* strains, there was no significant association between isolation year and PFGE pattern (Fig. 2B).

Discussion

A total of 134 GGS strains were isolated from bacteremia during the 18 years of surveillance. All EM non-susceptible strains belonged to the *S. dysgalactiae*, and the average EM non-susceptible rate was 40.3%. The annual EM non-susceptible rate was not significantly changed, whereas *ermB* and *mefA* were significantly increased and decreased, respectively. The *emmSTG840.0* and *emmSTC839.0* types were two dominant *emm* types, and significantly associated with *mefA* and *ermB*, respectively.

In this study, all strains belonging to the *anginosus* group were susceptible to EM. However, several studies found that *S. anginosus* were resistant to EM,[21–23] suggesting that the importance of the EM non-susceptible *anginosus* group cannot be overlooked. Furthermore, our study also supported the previous studies showing that *S. anginosus* was *emm* nontypable.[24,25]

The average EM non-susceptible rate in this study was 40.8%, which is much higher than the 7% in Hong Kong (isolated from bacteremia), 24% in central Taiwan (isolated from diverse human diseases), and 11.1% in Japan (isolated from severe infections).[10,18,26] Only GGS isolated from children in China had a similar rate.[9] Interestingly, in Taiwan, the EM non-susceptible rate of GAS was 40–70% before 2000.[27,28] However, since the usage of antibiotic was restricted after 2001, the EM non-susceptible rate of GAS was significantly decreased from 61% in 1998 to 17% in 2003.[29,30] However, the EM non-susceptibility in GGS was not significantly changed during the period. Although *S. dysgalactiae* is similar to GAS, and *S. dysgalactiae* was the major source of EM non-susceptibility, the mechanism leading to high EM non-susceptibility in GGS may be different from GAS.

The *emmSTG840.0*, *emmSTG485.0*, and *emmSTC839.0* types are the top 3 prevalent types in GGS causing bacteremia in this study. Similar to our results, *emmSTG840* and *emmSTG485* types were two dominant *emm* types found in strains causing bacteremia in Northern Taiwan and Jerusalem,[2,4] and *emmSTG840* was associated with recurrent bacteremia in Jerusalem.[4] Furthermore, *emmSTG840.0* and *emmSTC839.0* were associated with EM non-susceptibility in our study. Since these two *emm* types were associated with bacteremia and EM non-susceptibility, the importance of *emmSTG840.0* and *emmSTC839.0* types in human infection should be continuously monitored. The underlying mechanisms leading to bloodstream infections and antibiotic resistance also require further studies.

The prevalence of *ermB* and *mefA* in GGS significantly increased and decreased, respectively, in this study. In Taiwan, *ermB* was frequently found in the pA15 plasmid.[31] Whether increased prevalence of *ermB* in GGS was due to the presence of pA15 requires further study. Interestingly, DNA from 10 *ermB*-containing *emmSTC839.0* strains was not digested by *Sma*I. In GAS, the strain with prophage 10394.4 was resistant to *Sma*I digestion.[32] Whether prophage 10394.4 can be inserted into GGS requires further analysis. In *mefA*-containing *emmSTG840.0* strains, PFGE patterns A and B had significantly different temporal distribution, suggesting that the clonal shift from PFGE pattern A to B occurred in *emmSTG840.0* strains. Since 73% of *mefA*-containing strains were the *emmSTG840.0* type (Table 3), and strains with PFGE pattern B were less than those with PFGE pattern A (Fig. 2A), the clonal shift from PFGE pattern A to B may lead to a decrease in the prevalence rates of the *emmSTG840.0* type and *mefA* gene. In addition, two

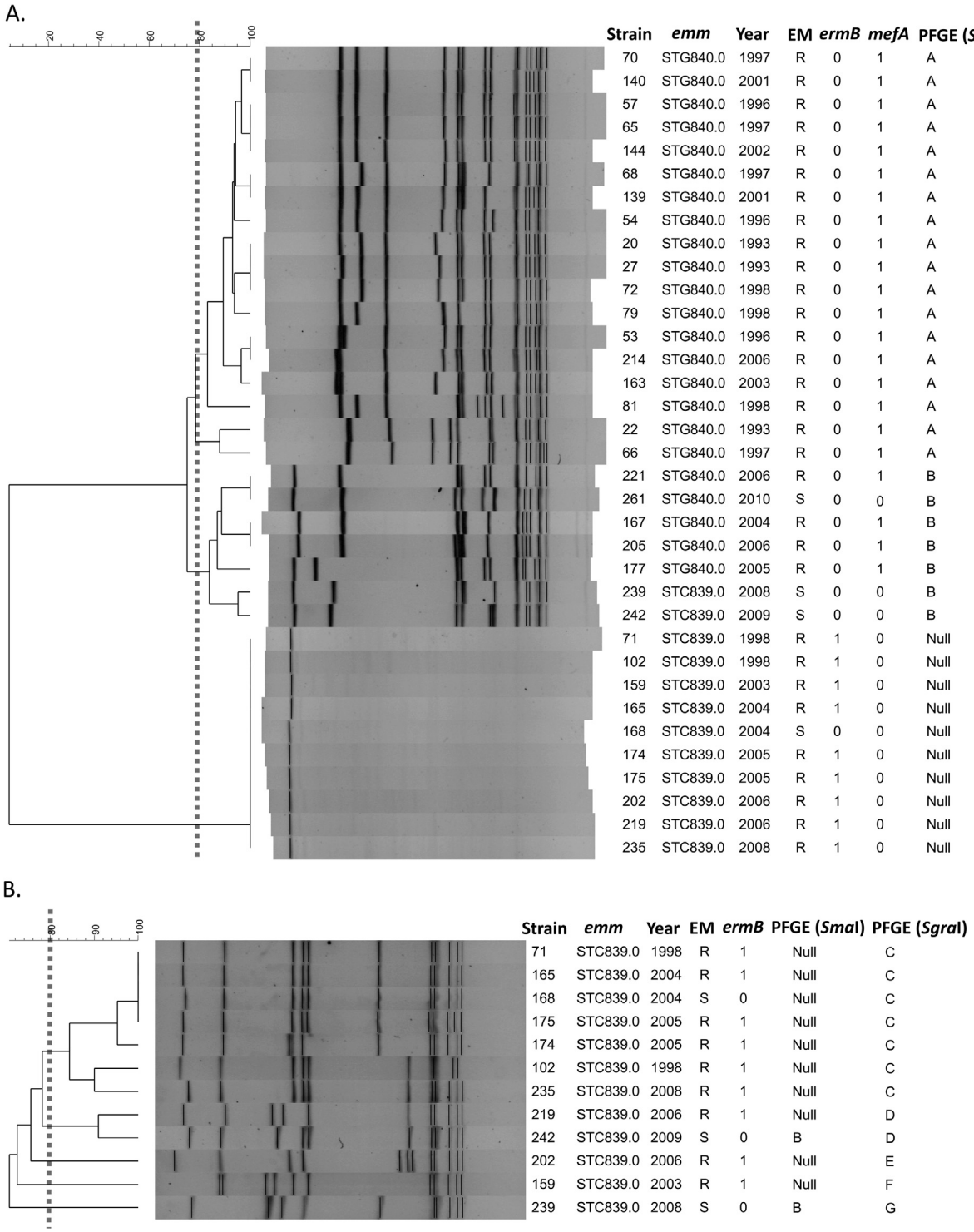

Figure 2. *Sma*I- (A) and *SgrA*I-digested (B) PFGE patterns of *emmSTG840.0* and *emmSTC839.0* GGS isolates. The strain number (Strain), *emm* type (*emm*), isolation year (Year), erythromycin susceptibility (EM), presence of *ermB* and *mefA*, and *Sma*I- or *SgaA*I-digested PFGE pattern are listed. The "R" and "S" indicate EM non-susceptible and susceptible, respectively. The "0" and "1" in the columns of *ermB* and *mefA* indicate the absence and presence of resistance genes, respectively. The dash line indicates 80% similarity.

strains did not have *ermA*, *ermB*, *ermC*, or *mefA*, suggesting that there are unknown mechanisms leading to EM non-susceptibility in GGS.

In conclusion, this longitudinal study of GGS causing bacteremia revealed high EM non-susceptibility in southern Taiwan. Since the *emmSTG840.0* and *emmSTC839.0* types were dominant, and associated with EM non-susceptibility, the underlying mechanism of how these two *emm* types lead to severe infections needs further elucidation. Constant surveillance is also warranted.

Conflicts of interest

The authors declare that they have no conflict of interest.

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