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ORIGINAL ARTICLE

Molecular characterization of Group A streptococcal isolates causing scarlet fever and pharyngitis among young children: A retrospective study from a northern Taiwan medical center

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Background/Purpose: Little information is available on the differences in frequency of pyrogenic exotoxin genes between strains of group A streptococci that cause scarlet fever and those that cause pharyngotonsillitis in children in Taiwan. This study retrospectively monitored the presence of pyrogenic exotoxin genes, the *emm* typing, and the susceptibility of macrolide drugs in *Streptococcus pyogenes* isolated from children diagnosed with scarlet fever and pharyngotonsillitis in northern Taiwan.
Materials and methods: Isolates of *S. pyogenes* were recovered from children with scarlet fever ($n = 21$) and acute pharyngotonsillitis ($n = 29$) during 2000–2011. The isolates were characterized according to the presence of *spe* genes and *emm* typing. Antibiograms were determined by the disk diffusion method and agar dilution test. Polymerase chain reaction was used to detect the presence of *erm* genes in isolates that showed nonsusceptibility to erythromycin. All isolates underwent additional genotyping by pulsed-field gel electrophoresis.
Results: In isolates from patients with scarlet fever, the frequencies of pyrogenic exotoxin genes were 9.5% for *speA*, 81.0% for *speB*, 4.8% for *speC*, and 71.4% for *speF*. In isolates from patients with pharyngotonsillitis, the frequencies were 17.2% for *speA*, 72.4% for *speB*, 13.8% for *speC*, and 69.0% for *speF*. There were no significant differences in frequencies of the exotoxin genes

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between the two groups of isolates. Eight *emm* sequence types were identified from all group A streptococci isolates. The most common types were *emm*12 followed by *emm*1 and *emm*4. The erythromycin resistant rate was 4/50 (8%). The *ermB* gene was detected in only one isolate from a patient with pharyngotonsillitis. Pulsed-field gel electrophoresis had a total of three sets of clustered strains, which showed >80% homology and belonged to the same *emm* type.
Conclusion: There were no significant differences in frequencies of the *spe* genes between *S. pyogenes* isolates from patients with scarlet fever and patients with pharyngotonsillitis. The most common *emm* type was *emm*12. Low erythromycin resistance in *S. pyogenes* was observed.

Introduction

Group A streptococci (GAS) cause a wide range of diseases including acute pharyngotonsillitis, impetigo, scarlet fever, acute rheumatic fever, acute glomerulonephritis, necrotizing fasciitis, and toxic shock syndrome.[1] Scarlet fever, characterized by the presence of a "strawberry tongue", sore throat, and sandpaper-like rash, normally occurs in children aged 4—7 years. During 1925—1950, scarlet fever was the dominant cause of death in childhood and adolescence in the United States. Although the epidemics eased after 1950, outbreaks of scarlet fever occurred sporadically.[2] In 2005, 1132 cases of scarlet fever were reported in Taiwan.[3] Due to less severe epidemics, scarlet fever has not been classified as a third-category notifiable diseases in Taiwan since October 2007.[4]

It is still unclear which virulence factors cause scarlet fever and which cause acute bacterial pharyngotonsillitis in pediatric patients. In addition to the various virulent constructive materials embedded on the surface, GAS also secrete several superantigen-like erythrogenic toxins including streptococcal pyrogenic exotoxin type A (*speA*), type B (*speB*), type C (*speC*), and type F (*speF*).[5] The M protein (*emm* typing), which extends from the cell wall and is anchored in the membrane, plays a potential role as another virulence factor in the streptococcal infections.[1] It has been proposed that streptococcal pyrogenic exotoxins produce the classic scarlet fever rash by enhancing acquired skin reactivity to streptococcal antigens.[6] Among scarlet fever associated strains, 45% contain the *speA* gene as compared to 15% among general GAS according to the World Health Organization.[7] In a study spanning more than five decades, Tyler et al have reported that 81.3% of the isolates associated with scarlet fever carried the *speA* gene, whereas only 18.4% of isolates from patients with pharyngitis carried the gene.[8] The exotoxin *speA* and *speC* genes are transferred by a laterally transmissible bacteriophage.[9] In addition to pyrogenicity, *speB* acts as a cysteine protease.[10,11] *SpeF*, similar to *SpeA* and *SpeB*, induces massive amounts of interferon-γ and tumor necrosis factor-β, with weak T helper 2 cytokine responses.[1] Results from previous investigations have shown that temporal and geographic variations in *spe* genes are responsible for the fluctuations in disease character.[12] Information about the types of exotoxins associated with scarlet fever or pharyngotonsillitis is poorly understood in northern Taiwan communities. The aim of the present study was to unravel the distribution of *spe* genes among isolates of GAS as well as to compare the frequency of these exotoxin genes between isolates from patients with scarlet fever and those from patients with pharyngotonsillitis.

Materials and methods

GAS strains

During November 2000 to October 2011, a total of 50 non-duplicate isolates of GAS were obtained from throat cultures from patients aged <18 years who were prepared to be treated for scarlet fever ($n = 21$) or pharyngotonsillitis ($n = 29$) at the Tri-Service General Hospital, a 1400-bed tertiary medical center in northern Taiwan. Clinical data on all patients were obtained from the Clinical Microbiology Laboratory and included age, sex, day of disease onset, and symptoms. The patients who presented with a "strawberry tongue", sandpaper rash, and sore throat were followed up 1 week later in the second outpatient service. Scarlet fever was confirmed if the rash began to fade and was followed by desquamation. The clinical diagnosis of pharyngotonsillitis was based on the principal complaints of fever and sudden onset of sore throat or more of the following signs: inflammation of the pharynx and tonsils with exudates or cervical lymphadenopathy. The isolates were preserved at −70°C in Todd—Hewitt medium (BBL; Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 15% glycerol until use. Isolates were initially identified on the basis of hemolytic colony morphology and bacitracin susceptibility. In isolates that were initially unidentifiable, identification was established with the pyrrolidonyl arylamidase test and the streptococcal grouping kit (Oxoid; Basingstoke, Hampshire, UK).

Antibiotic susceptibility test

In vitro susceptibility test of the isolates was performed by Kirby—Bauer agar disc diffusion method. *Streptococcus pneumoniae* ATCC 49619 was used for quality control. The following antibiotics were tested: chloramphenicol (30 μg), penicillin G (10 μg), ampicillin (10 μg), clindamycin (2 μg), erythromycin (15 μg), and vancomycin (30 μg) (BBL; Becton, Dickinson and Company). Once the inhibition zone diameter of erythromycin was <15 mm, the minimal inhibitory

concentration (MIC) of erythromycin was determined on the resistant isolates with the plate dilution method of Mueller–Hinton agar.[13] The test agar was supplemented with 5% sheep blood. An inoculum of 10^4–10^5 colony forming units per milliliter was used. The results were read after 24 hours in the 5% CO_2 incubator at 35°C.

Double-disk diffusion test

The double-disk diffusion test was performed according to the protocol described by Martin et al.[14] The method can help in identifying the mechanism of erythromycin resistance by distinguishing among different resistance phenotypes of GAS strains; namely, cMLS (constitutive macrolide–lincosamide–streptogramin B resistance phenotype), iMLS (inducible macrolide–lincosamide–streptogramin B resistance phenotype), and the M phenotype (resistant to macrolides, but susceptible to lincosamide and streptogramin B antibiotics). Suspensions of GAS that had been adjusted to an equivalent 0.5 McFarland standard (approximately 1.5×10^8/mL) were inoculated onto Mueller–Hinton agar containing 5% sheep blood. An erythromycin disk (30 μg/mL) and a clindamycin disk (10 μg/mL) were placed 15–20 mm apart (edge to edge) on each plate. Resistance to both erythromycin and clindamycin indicated a cMLS phenotype; resistance to erythromycin, characterized by the presence of blunting of the zone of inhibition around the clindamycin disk on the side of the erythromycin disk, indicated an iMLS phenotype; and resistance to erythromycin and susceptibility to clindamycin, characterized by blunting of the zone around the erythromycin disk, was indicative of the M phenotype.

Determination of spe and erm genes

Fresh isolates were grown overnight on 5% sheep blood agar plates at 37°C. A filled loop of each strain was added to 50 μL lysis buffer (20 mM Tris–HCl; pH 8.0; 100 mM NaCl; 0.1 mM EDTA). The mixture was boiled at 100°C for 5 minutes, and cooled to 37°C for 15 minutes. It was centrifuged at 12,000 rpm (relative centrifugal force: 13,353.98 *g*) with the centrifuge MRB099J18 (Beckman Coulter, Indianapolis, IN, USA) for 5 minutes. The supernatant was recovered for DNA template. Polymerase chain reaction (PCR) was used to amplify the *speA*, *speB*, *speC*, and *speF* genes as previously reported.[15,16] Amplification was performed in a DNA thermal cycler (Eppendorf Mastercycler Thermal Cycler; Eppendorf AG, Hamburg, Germany) with the following conditions: for *speA*, *speB*, and *speC* genes: one cycle at 94°C for 3 minutes; 30 cycles at 94°C for 1 minute; 62°C for 1 minute; 72°C for 1 minute; and 72°C for 7 minutes. For the second PCR of *speA*, *speB*, and *spec* genes: one cycle at 94°C for 3 minutes; 19 cycles at 94°C for 1 minute; 60°C for 1 minute; 72°C for 1 minute; and 72°C for 7 minutes. For *speF*, *ermA*, *ermB*, and *ermC* genes: one cycle at 94°C for 3 minutes; 19 cycles at 94°C for 1 minute; 60°C for 1 minute; 72°C for 1 minute; and 72°C for 7 minutes. The presence of *ermA*, *ermB*, and *ermC* in erythromycin-resistant isolates was detected using PCR as described by Sutcliffe et al.[17] The sizes of two-step PCR products for *speA* were 818 bp and 500 bp; 1106 bp and 912 bp for *speB*; and 801 bp and 654 bp for *speC*. The expected sizes of PCR products were 645 bp for *ermA*, 639 bp for *ermB*, and 642 bp for *ermC*.

emm typing

The protocol described at the Centers for Disease Control and Prevention (CDC) website (http://www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm) was used to prepare *emm* DNA fragments for nucleotide sequence determination. The *emm* types were assigned from the database at the CDC website (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome).

Pulsed-field gel electrophoresis

Plug slices (width, 2 mm) were digested overnight with 10 U restriction enzyme *Sma*I (New England Biolabs, Beverly, MA, USA). DNA fragments were separated on 1% Pulsed Field Certified Agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5× TBE buffer (45 mM Tris–HCl, 45 mM boric acid, and 1.0 mM EDTA; pH 8.0) at 14°C. Lambda Ladder PFG Marker, N0340S (New England Biolabs) was used in this analysis for gel normalization. Pulsed-field gel electrophoresis (PFGE) was performed using a CHEF Mapper XA system (Bio-Rad Laboratories) for 26.9 hours, with initial and final switching times of 1.79 seconds and 54.17 seconds, respectively, as reported previously.[18] The range was between 25 kb and 600 kb. PFGE images were analyzed using the fingerprint analysis software BioNumerics, version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). Strains sharing identical or closely related PFGE profiles with similarity coefficients >80% and the same *emm* type were considered to be the similar strain type.

Statistical analysis

Data were analyzed with the Fisher's exact test or χ^2 test as appropriate. Statistical analyses were performed with the statistical package SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). A p value ≤ 0.05 was considered to represent statistical significance.

Results

Distribution of exotoxin and emm genes

Only seven of the 50 GAS isolates carried the *speA* gene. Among the seven isolates positive for *speA*, two were obtained from patients with scarlet fever and five from patients with pharyngotonsillitis. The *speB* gene was detected in 17 isolates from patients with scarlet fever and in 21 isolates from patients with pharyngotonsillitis; the *speC* gene was found in one isolate from a patient with scarlet fever and in four isolates from patients with pharyngotonsillitis; and the *speF* gene was detected in 15 isolates from patients with scarlet fever and in 20 isolates from patients with pharyngotonsillitis. The frequencies of *speA*, *speB*, *speC*, and *speF* were similar among two groups

Table 1 Baseline characteristics and distribution of *speA*, *speB*, *speC*, *speF*, and *ermB* of 50 isolates from pediatric patients

	Scarlet fever	Acute pharyngotonsillitis	
No. of strains isolated from patients	21	29	
No. of boys	12 (57.1%)	14 (48.3%)	$p = 0.89$[a]
Average age (yr) ± SD	6.0 ± 2.8	9.8 ± 3.3	$p < 0.01$[b]
speA	9.5% (2/21)	17.2% (5/29)	$p = 0.68$[d]
speB	81.0% (17/21)	72.4% (21/29)	$p = 0.72$[a]
speC	4.8% (1/21)	13.8% (4/29)	$p = 0.38$[d]
speF	71.4% (15/21)	69.0% (20/29)	$p = 1.00$[a]
ermB[c,e]	0% (0/2)	50% (1/2)	$p = 1.00$[d]

[a] χ^2 test.
[b] Student *t* test.
[c] The value was number of positive/number of erythromycin-resistant isolates.
[d] Fisher's exact test.
[e] No detectable *ermA* and *ermC* genes appeared in either group.
SD = standard deviation.

of isolates (Table 1). Eight *emm* sequence types were identified from all the GAS isolates. The most common types were *emm*12 (58.0%), followed by *emm*1 (24.0%), and *emm*4 (8.0%). Other less common types included *emm*6 (2.0%), *emm*22 (2.0%), *emm*28 (2.0%), *emm*77 (2.0%), and *emm*89 (2.0%).

Antimicrobial susceptibility

All of the isolates in this study were susceptible to penicillin, ampicillin, and vancomycin. However, we found that 4.8% of isolates from patients with scarlet fever and 10.3% of isolates from patients with pharyngotonsillitis were resistant to chloramphenicol and clindamycin. In addition, we found that 9.5% of isolates from patients with scarlet fever and 6.9% of isolates from patients with pharyngotonsillitis were resistant to erythromycin during the study period. Among the four clinical isolates resistant to erythromycin in both groups, only one isolate (MIC for erythromycin: 256 μg/mL), which was collected from a patient with pharyngotonsillitis, possessed the *ermB* gene (Table 1). This strain appeared as constitutive MLS in the double-disk diffusion test. The other three strains (MIC for erythromycin: 128 μg/mL, 128 μg/mL, and 64 μg/mL, respectively), two of which were isolated from scarlet fever, were shown to have the M phenotype of erythromycin resistance. The four erythromycin-resistant isolates were negative for both *ermA* and *ermC*.

PFGE typing

The PFGE-typed isolates were divided into 22 pulsotypes (Fig. 1). A total of three sets (A, B, and C) of clustered strains showed >80% homology and the same *emm* type. Cluster A and Cluster C were predominant among them. Cluster A had 10 strains, and two (20.0%) of them were from patients with scarlet fever. In Cluster B, the percentage of strains from scarlet fever patients was 33.3%. Cluster C had 23 strains, and nine (39.1%) of them were found in patients with scarlet fever. Strains in the three clusters were not associated with the two diseases. Each cluster was associated with a predominant *emm* type (*emm*1: Cluster A, *emm*4: Cluster B, *emm*12: Cluster C). Although the PFGE pattern of Strains 605, *329, *439, 125, 2, 28, 327, 374, 521, and 530 was identical, only Strain *439 had a different *emm* type among them. Strains *424, 630, *574, *592, *635, *76, and *95 had the same pulsotype. However, strain *574 was *emm*1, *95 was *emm*6, and the other five belonged to *emm*12. Moreover, none of the strains were duplicated.

Discussion

Streptococcal pyogenic exotoxins play a pathogenic role in GAS-related diseases.[4] The character of the exotoxins that cause invasive diseases varies between countries. To the best of our knowledge, no studies have investigated the correlation between virulence factors of GAS and streptococcal diseases in pediatric patients in Taiwan. In this study, we assessed the frequency of *spe* genes, compared the genotypes, and evaluated the rate of resistance to a variety of antimicrobial agents among GAS isolates that had been collected from patients with scarlet fever or pharyngotonsillitis in northern Taiwan during an 11-year period.

In the early 1990s, Iwasaki et al[19] and Norrby-Teglund and Kotb[5] revealed that most GAS strains carry the *speB* and *speF* genes, whereas *speA* and *speC* occur less frequently. Yu and Ferretti reported that 45% of scarlet-fever-associated GAS strains carried the *speA* gene and that only 15% of the total GAS strains carried the gene for that exotoxin.[7] By contrast, Hsueh et al reported that the strains responsible for an outbreak of scarlet fever at a hospital daycare center in Taiwan did not carry the *speA* gene.[20] In our study, we did not find a significant difference in the distribution of *speA* genes between isolates obtained from children with scarlet fever and patients with pharyngotonsillitis. Nandi et al found that the presence of the *speA* gene was usually associated with scarlet fever or toxic shock-like syndrome.[21]

Other reports have noted that the less potent *speB* and *speC* toxins are sometimes present, either individually or together, in such strains, posing a question regarding the role of *speA* as a major virulence factor.[22] The exotoxin genes *speB*, *speC*, and *speF* have been reported in invasive GAS disease.[8,23,24] Studies have also shown that the majority of GAS contain the *speB* and *speF* genes, suggesting that both are chromosomally encoded.[20] In addition, *speC* has been shown to appear in conjunction with *speA* significantly more frequently in isolates from patients with scarlet fever than in those from patients with pharyngitis.[8,25]

Furthermore, we found that there was no significant difference in the frequency of the *speA*, *speB*, *speC*, and *speF* genes between isolates from children with scarlet fever and those from children with pharyngotonsillitis. This finding differs markedly from that reported in several

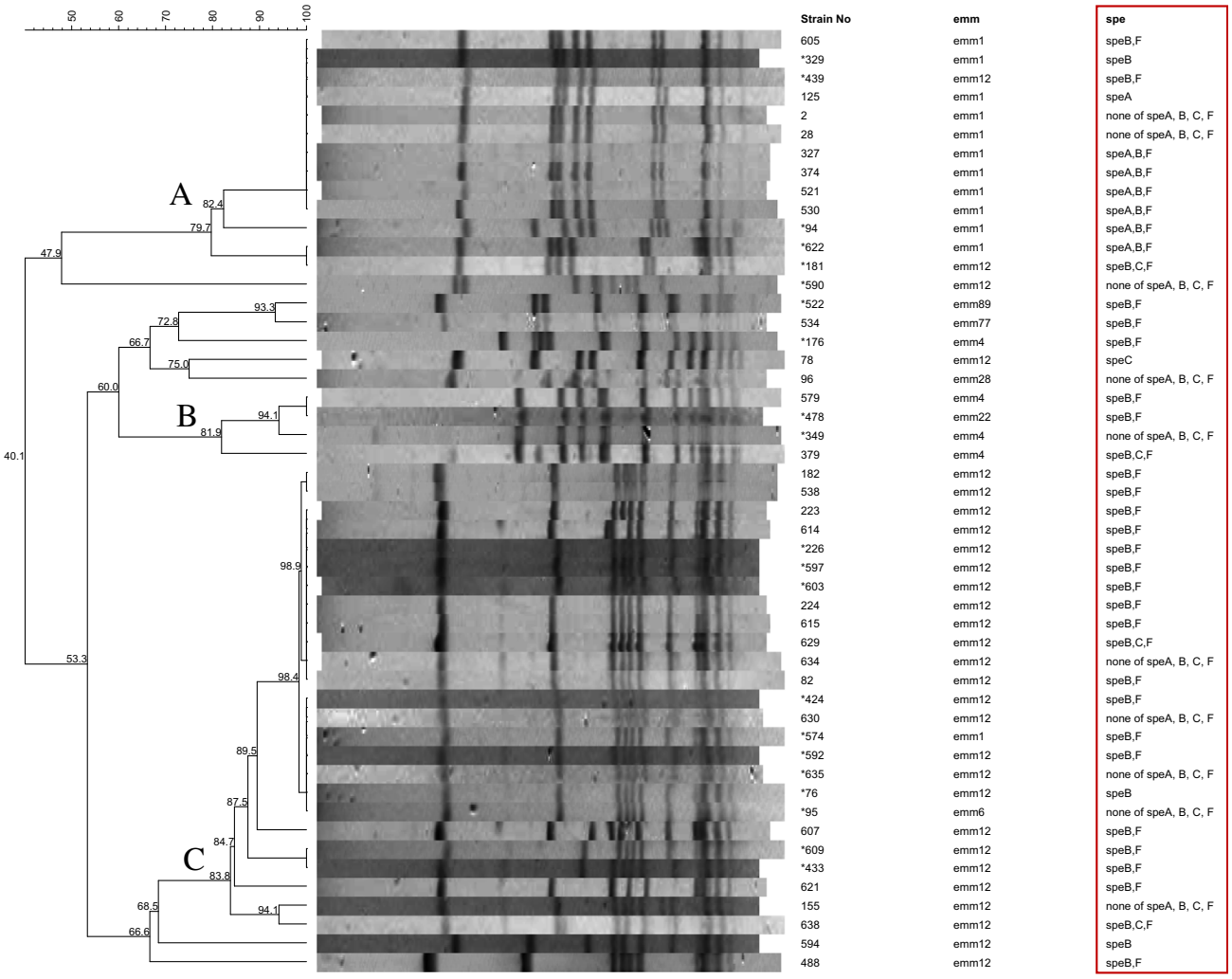

Figure 1. Dendrogram, *spe* patterns, and *emm* types of all the group A streptococci. The scale indicates the level of pattern similarity. The dendrogram was generated by use of the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm on the basis of Dice similarity coefficients >80%, with 1% optimization and 1.5% position tolerance.[29] Cluster A: 605, *329, 125, 2, 28, 327, 374, 521, 530, *94; Cluster B: 579, *349, 379; and Cluster C: 182, 538, 223, 614, *226, *597, *603, 224, 615, 629, 634, 82, *424, 630, *592, *635, *76, 607, *609, *433, 621, 155, 638. Asterisk points out the Isolates from scarlet fever patients.

previous studies.[6,7,20] The outcome of GAS infection is not only strain related, but is also related to a combination of several factors, such as host immunity and exotoxin production.[26] Bacterial characteristics may also play a pathogenic role in severe streptococcal infections, because GAS without the superantigen genes *speA* and *speC* have been shown to cause invasive disease.[27]

In addition to streptococcal pyrogenic exotoxin, M phenotype is one of the gold standards for the characterization of streptococcal virulence. In the present study, a total of eight *emm* types were sequenced, and the major types were *emm*12, *emm*1, and *emm*4. The distribution was slightly different from those reported in the adult population in Taiwan. Hsu and Wu reported that *emm*1 and *emm*4 were most frequent, harbored by 56 (72.7%) isolates and 20 (26.0%) isolates, respectively, among 77 GAS isolates associated with scarlet fever epidemics occurring between 1993 and 2002 in southern Taiwan.[28] Chen et al revealed the most common *emm* types were *emm*1 (29.2%), *emm*4 (24.1%), *emm*12 (19.0%), *emm*6 (15.8%), stIL103 (5.7%), and *emm*22 (1.9%) among 830 *S. pyogenes* isolates collected between 2001 and 2002 from patients with scarlet fever in northern Taiwan.[29] Yan et al showed that *emm*1, *emm*4, and *emm*25 were highly associated with scarlet fever at a Taiwanese University Hospital between 1993 and 2002.[30] Chiou et al found that *emm*4 (45%), *emm*12 (36%), *emm*1 (8%), and *emm*22 (7%) were the prevalent types frequently associated with scarlet fever in central Taiwan from 1996 to 1999.[31] Su et al indicated that the most frequent *S. pyogenes emm* types isolated at the National Cheng Kung University Hospital from 1998 to 2007 were type 12 (43.4%), type 4 (18.2%), and type 1 (16.9%).[32] Changes in M serotype distribution within different regions over time are probably due to prevalent M serotype immunity patterns within the populations, combined with the introduction of new M serotypes to these populations.[29] In the present survey, the identical PFGE pattern could be divided into different *emm* types. By contrast, the same *emm* type had different PFGE types. Therefore, the combination of these two methods could achieve better classification.

There is no obvious penicillin-resistant GAS at the present time.[33] An incremental increase in GAS strains that are resistant to erythromycin has been reported in Taiwan, Europe, and the United States.[34–36] One of the main resistance mechanisms is constructive change induced by methylation of the 50S ribosomal subunit.[37] Both the constitutive MLS and inducible MLS phenotypes are representative of this mechanism. The M phenotype represents a resistance pattern mediated by an efflux system encoded by *mefA*.[38] M phenotype (3/4, 75%) was more predominant than constitutive MLS phenotype (1/4, 25%) in our present survey. In southern Taiwan, Hsu and Wu found that most (94.8%) GAS strains with the constitutive MLS phenotype contained the *ermB* gene, and that almost 70% of erythromycin-resistant GAS strains had the M phenotype. They concluded that overuse of antibiotics to treat viral upper respiratory tract infections was the major contributor to high macrolide resistance (40–70%) in Taiwan.[28] In February 2001, the National Health Insurance system in Taiwan started to restrict reimbursement for antibiotics that are administered to treat upper respiratory tract infections without evidence of bacterial involvement. The low incidence of erythromycin resistance (8%) in this retrospective study was also observed in a study by Hsueh et al.[39] Most of the GAS strains (92%) were susceptible to erythromycin. If there is indeed a trend of erythromycin-sensitive strains in Taiwan, then that antibiotic might be the drug of choice for GAS infections in penicillin-hypersensitive patients.

In this study, we concluded that there was a lack of association between the presence of *speA*, *speB*, *spec*, or *speF* in the isolates from patients with scarlet fever and pharyngotonsillitis in northern Taiwan during the past 11 years. Each cluster contained both disease patterns. Continuous sampling of more isolates in this region is necessary, and further molecular typing should be conducted to confirm the phenomenon.

This study had several limitations. First, the case number was too small. Although scarlet fever cases are not sparse in Taiwan, sensitive rapid streptococcal antigen detection tests give less opportunity to collect a throat culture to analyze the distribution of streptococcal pyogenic exotoxin genes. Second, insufficient epidemiological data for *S. pyogenes* were collected. Third, no comparison of the exotoxin gene profile between invasive and noninvasive diseases was presented. To the best of our knowledge, this is the first study to analyze streptococcal pyogenic exotoxins in GAS isolates from children with scarlet fever in Taiwan. Further studies on other virulence factors including *emm* typing are necessary to elucidate the pathogenic mechanisms mediating GAS diseases, such as scarlet fever.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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