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ORIGINAL ARTICLE

The trend of macrolide resistance and *emm* types of group A streptococci from children at a medical center in southern Taiwan

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Background: Group A streptococcus (GAS) is a common pathogen in children. Macrolide resistance in GAS has been described worldwide. The aims of this study are to analyze macrolide resistance of GAS isolates in southern Taiwan and to clarify the relationship of *emm* typing and macrolide resistance in the past decade.
Methods: All GAS isolated from patients younger than 18 years at a single tertiary center in southern Taiwan were collected from 2000 to 2012. Antibiotics susceptibility to erythromycin, azithromycin, and clindamycin were determined by agar dilution method, and were interpreted by Clinical and Laboratory Standards Institute (CLSI) standards. *emm* typing was performed by polymerase chain reaction (PCR).
Results: A total of 301 isolates were collected during the period of 13 years. Scarlet fever (38.5%) and acute pharyngitis (32.2%) were the most common diagnosis. Decreased resistance rate of erythromycin from 53.1% in 2000 to 0% in 2010 was found, but it increased rapidly to 65% in 2011. The resistance rate of azithromycin was the lowest (4.2%) in 2005, but was higher than 15% after 2006. The involvement of the erythromycin resistance genes were *mef*A (53.1%), *erm*B (35.9%), and *erm*TR (10.9%). The resistance of clindamycin also increased since

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2011. *emm*12 was the most common serotype and accounted for 44.9% of all isolates. Compared with the non-*emm*12 group, resistance to erythromycin, azithromycin, and clindamycin were more frequently detected in the *emm*12 group.
Conclusion: Increased resistance of GAS to macrolide and clindamycin was found in recent years. *emm*12 was the main serotype for macrolide resistance.

Introduction

Group A Streptococcus (GAS, or *Streptococcus pyogenes*) causes a wide range of clinical illness, including pharyngotonsillitis, scarlet fever, sepsis, and toxic shock syndrome in children. Penicillin is the first-line drug of choice for the treatment of GAS infection. Erythromycin is an alternative choice in persons who are allergic to penicillin. Azithromycin, which is given in a daily dose and short course, is widely used in clinical practice for the treatment of respiratory tract infection, such as *Mycoplasma pneumoniae* infection.

However, macrolide resistance had been described worldwide since the 1990s. In Finland, the resistance rate of GAS to erythromycin from blood cultures increased from 4% in 1988 to 24% in 1990, and the erythromycin resistance was associated with the consumption of macrolide.[1,2] In the United States, a high erythromycin resistance rate (48%) was reported in schoolchildren in Pittsburgh between 2000 and 2001.[3]

In Taiwan, Hsueh et al[4] reported decreased susceptibility of erythromycin to GAS in 1992 and 1993. They demonstrated decreased erythromycin resistance of GAS in 1999 to 2003 from 46% to 17%.[5] The relationship was found between decreased resistance rate and decreased erythromycin consumption because of governmental policy in the restriction of antibiotics treatment in Taiwan. However, there are no available data for the susceptibility rate in recent years in Taiwan.

The aims of this study are to analyze macrolide resistance of GAS isolates at a medical center in southern Taiwan and to clarify the relationship of *emm* typing and macrolide resistance in the past decade.

Materials and methods

Patient enrollment and definition

All GAS isolates were collected from 2000 to 2012 from patients younger than 18 years at a 1200-bed tertiary medical center, National Cheng Kung University Hospital (NCKUH) in southern Taiwan. Demographic data, including age, sex, diagnosis, and culture sites were reviewed. The diagnosis is based on the following definitions. Acute pharyngitis was defined as clinical symptoms such as fever and sore throat or injected pharynx on physical examination. Scarlet fever was defined as fever with strawberry appearance of the tongue, and diffuse erythematous rash with sandpaper-like consistency. Cellulitis was defined as inflammation of the skin and subcutaneous tissue surrounding the infection site. Necrotizing fasciitis was defined as infection over the deep subcutaneous tissue and fascia with rapid progression. Streptococcal toxic shock syndrome was defined according to the definition from The Working Group on Severe Streptococcal Infections.[6]

In a single patient with multiple isolates from different sites, only the isolate from a sterile site, such as blood or pus, was included. Isolates with culture report showed *S. pyogenes* initially, but *emm* typing showed other group streptococcus were also excluded.

All informed consent forms and relevant study-related documentation were approved by a third-party Institutional Review Board.

Microbiologic features

Antibiotics susceptibility was determined by agar dilution method with Mueller Hinton agar supplemented with 5% sheep blood. The minimum inhibitory concentrations (MICs) of erythromycin, azithromycin, and clindamycin were recorded, and the MICs were interpreted according to the criteria from the Clinical and Laboratory Standards Institute (CLSI).[7] The MICs of erythromycin $\leq$0.25 μg/mL was susceptible, 0.5 μg/mL was intermediate, and $\geq$1.0 μg/mL was resistant. As for azithromycin, cut points of susceptible, intermediate, and resistant isolates were $\leq$0.5 μg/mL, 1.0 μg/mL, and $\leq$2.0 μg/mL, respectively. The cut points of susceptible, intermediate, and resistant isolates were $\leq$0.25 μg/mL, 0.5 μg/mL and $\geq$1.0 μg/mL, respectively, for clindamycin. MIC_{50} and MIC_{90} of isolates to erythromycin, azithromycin, and clindamycin were also measured every year. Isolates with intermediate susceptibility to azithromycin (MIC = 1.0 μg/mL) were rechecked with E-test.

emm typing

Polymerase chain reaction (PCR) amplification of the *emm* gene was performed with previously described primers.[8] The amplicons were sequenced on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with the primer pair for PCR. The *emm* sequences obtained were interpreted according to the database established by the United States Centers for Disease Control and Prevention (CDC).[9] An isolate was considered to be of a given *emm* type if it had >98% identity over the first 180 bases obtained.

Detection of erythromycin resistance genes

PCR assay is used to identify the genetic mechanism of resistance. The *mef*A, *erm*B, and *erm*TR resistance genes of

isolates with erythromycin MIC $\geq$0.5 μg/mL were detected by PCR amplification with the use of previously described primers.[10,11] GAS isolates that were susceptible to erythromycin, obtained during the study period, were used as negative controls. Positive control strains yielding the expected products sizes of were 348 bp, 639 bp, and 206 bp for *mef*A, *erm*B, and *erm*TR, respectively.

Pulse-field gel electrophoresis analysis

The pulse-field gel electrophoresis (PFGE) protocol for GAS was developed on the basis of Gautom's *Escherichia coli* rapid PFGE protocol, with minor modifications.[12] Briefly, the individual GAS colonies were grown on blood agar plates and incubated in 5% CO_2 at 37 °C. Following suspension in suspension buffer (100 mM Tris and 100 mM EDTA) and washing, the bacterial pellet was resuspended in suspension buffer. An agarose preparation [1% Seakem Gold agarose (FMC Bio-Products, Rockland, Maine, USA) with 1% sodium dodecyl sulfate (SDS) in Tris-EDTA buffer (10 mM Tris and 1 mM EDTA, pH 8)] was mixed with each suspension. The bacterium-agarose mixture was then added to plug molds (Bio-Rad Laboratories, Hercules, CA, USA). After solidification, the plugs were transferred to centrifugation tubes containing lysis buffer (50 mM Tris, 50 mM EDTA, 1% sodium lauryl sarcosine, 0.5 mg proteinase K; pH 8) and incubated at 50 °C for 2 hours. After the completion of the agarose plug preparation, plug slices were made and digested with *Sma*I. The DNA fragments were separated by electrophoresis in 1% agarose gels at 14 °C in Tris-borate-EDTA buffer (pH 8). The gels were stained with ethidium bromide, destained with water, exposed on a UV transilluminator, and photographed. BioNumerics 6.5 (Applied Maths, Sint-Martens-Latem, Belgium) was used to analyze PFGE images, and isolates with the same *emm* type sharing PFGE pattern with >80% similarity were considered to be similar strains.

Statistical analysis

All statistical analyses were conducted using the SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Data were analyzed to determine the statistical significance of differences between antibiotics resistance and *emm* typing. A p value <0.05 was considered statistically significant.

Results

Demographic data

A total of 301 isolates were collected during a 13-year period (range: 7-46 isolates/y). The mean age of patients was 7.6 ± 3.4 years (6 days old-17.7 years old), and male sex was predominant (58.5%). Scarlet fever (38.5%) and acute pharyngitis (32.2%) were the most common diagnosis. Overall, 19 patients (6.3%) had severe infection with streptococcal toxic shock syndrome (4.3%), sepsis (3.7%), or necrotizing fasciitis (1.3%). Most of the isolates (70.8%) were obtained from the throat, followed by pus or a wound. Eleven (3.7%) isolates were obtained from blood.

Susceptibility and $MIC_{50/90}$

The resistance rates of erythromycin were high in 2000 (53.1%) and 2001 (57.1%), but it decreased rapidly to 15.6% in 2002 (Fig. 1). Resistance rates were lower than 25% between 2002 and 2010, and were 0% in 2009 and 2010. However, it increased rapidly to 65% in 2011, and 44.4% in 2012. As for azithromycin, high resistance rates (>50%) were also found in 2000 and 2001. It decreased to lower than 25% in the following 5 years, which is the same as erythromycin, and the lowest resistance rate of azithromycin was 4.2% in 2005. But it increased rapidly to 59.1% in 2007 and was higher than 30% after 2010. Clindamycin had a low resistance rate below 10% for many years. However, similar to erythromycin and azithromycin, the resistance rate of clindamycin increased rapidly after 2011.

Overall, 63 isolates (20.9%) were resistant to erythromycin, 82 isolates (27.2%) were resistant to azithromycin, and 33 isolates (11.0%) were resistant to clindamycin. Sixty-one isolates in 63 erythromycin-resistant strains (96.8%) were also resistant to azithromycin, and all clindamycin resistant isolates were resistant to azithromycin. In azithromycin-resistant strains, 61 isolates (74.4%) were resistant to erythromycin, and 33 isolates (40.2%) were resistant to clindamycin.

Regarding MIC_{90} of erythromycin, we found that the MIC level was high prior to 2001 (MIC_{90}: 256 μg/mL in 2000, 16 μg/mL in 2001), then it decreased gradually since 2003 (Fig. 2). Another minor elevation of MIC_{90} was found during 2007 and 2008, then it declined in the following 2 years. However, there was a rapid increase since 2011 (MIC_{90}: 128 μg/mL). As for azithromycin, the MIC_{90} level was also high prior to 2001, and it decreased in the following years. The MIC_{90} of azithromycin, erythromycin, and clindamycin were significantly increased after 2010. The MIC_{90} of azithromycin was higher than erythromycin and clindamycin. The MIC_{50} of azithromycin, erythromycin, and clindamycin also peaked in 2011 (data are not shown).

emm typing and resistance

In total, 23 different *emm* types were identified. *emm*12 was the most common serotype, which accounted for 44.9% of all isolates (n = 135), followed by *emm*1 (19.6%) and *emm*4 (17.9%). *emm*12, *emm*1, and *emm*4 account for 82.4% of all isolates.

The distribution of *emm* types by year are shown in Fig. 3. *emm*12 is the leading serotype in 8 years, and were predominantly more than other types (>50%) in 2000, 2001, 2005, 2006, 2011, and 2012. *emm*1 were predominant in 2002 and 2003, and *emm*4 were predominant in 2004 and 2008.

In those years when *emm*12 were predominant, the antibiotics resistance rates were relatively higher. In comparison with the non-*emm*12 group, *emm*12 was found to have higher resistance to erythromycin (28.1% vs. 15.1%, p = 0.004), azithromycin (39.3% vs. 17.5%, p < 0.001), and clindamycin (19.3% vs. 4.2%, p < 0.001).

Erythromycin resistance genes

Erythromycin resistance genes with *mef*A, *erm*B, *erm*TR were detected in 65 erythromycin nonsusceptible isolates

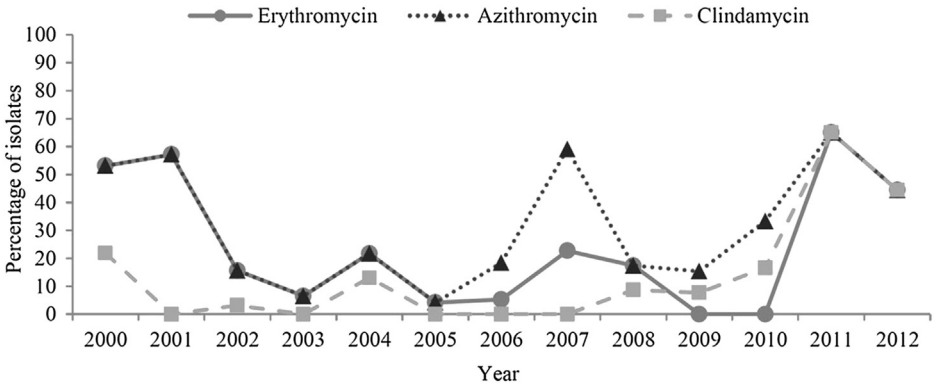

Figure 1. Antibiotic resistance rates of group A streptococci to erythromycin, azithromycin, and clindamycin between 2000 and 2012 at National Cheng Kung University Hospital in Tainan City, Taiwan.

(MIC $\geq$ 0.5 μg/mL). We found at least one resistance gene in 63 isolates. Two genes (*mef*A, *erm*B) were detected in one isolate. No resistance gene was detected in two isolates. Overall, *mef*A accounted for 53.1% (n = 34), *erm*B accounted for 35.9% (n = 23), and *erm*TR accounted for 10.9% (n = 7). Fig. 4 showed the resistance gene distribution by year. *mef*A gene was predominant prior to 2008, and *erm*B gene was predominant after 2011.

PFGE analysis

Increased macrolide resistant *emm*12 isolates were detected in 2011 and 2012. Six erythromycin-resistant *emm*12 isolates in 2011 and three in 2012 were collected randomly to perform the PFGE study. The result showed almost the same patterns among these nine isolates with more than 90% similarity (Fig. 5).

Discussion

A macrolide-resistant strain of GAS has been reported worldwide and has been an ongoing problem since the 1990s. A nationwide study in Finland showed a steady decline in erythromycin resistance rate, from 16.5% in 1992 to 8.6% in 1996.[2] This decline was related to reductions in the use of macrolide for respiratory and skin infection in outpatients by nationwide recommendation. Another study further confirmed the relationship between decreased macrolide consumption and declined erythromycin resistance. During 1997–2001, the regional erythromycin resistances of GAS in Finland were related to the macrolide consumption of the previous year.[13]

In the United States, the macrolide resistance among GAS was low prior to 2000. But high resistance rate to erythromycin was found between 2000 and 2001.[3] It was reported that 48% of isolates from school children were resistant to erythromycin, and 38% of isolates obtained from the community were resistant to erythromycin. This outbreak was due to a single strain, and *emm*6 was the major resistant strain. A large nationwide study showed 129 of 1885 isolates (6.8%) were erythromycin resistant, 130 (6.9%) were azithromycin resistant, and 10 (0.5%) were clindamycin resistant between 2002 and 2003.[14] *emm* typing showed the 129 erythromycin-resistant strains belonged to 44 PFGE patterns and 28 *emm* types.

In France, the erythromycin resistance rate was 22.4% in isolates collected from children age 2–16 years with acute pharyngitis between 2002 and 2003.[15] In one study performed in Portugal during 2000–2006, the asymptomatic colonization rate was 10.7% in children. The macrolide resistance rate was 16.5%, whereas the rate was higher among children age 0–6 years (18.2%) and among adults (16.2%) than among children age 7–16 years (8.4%).[16] In

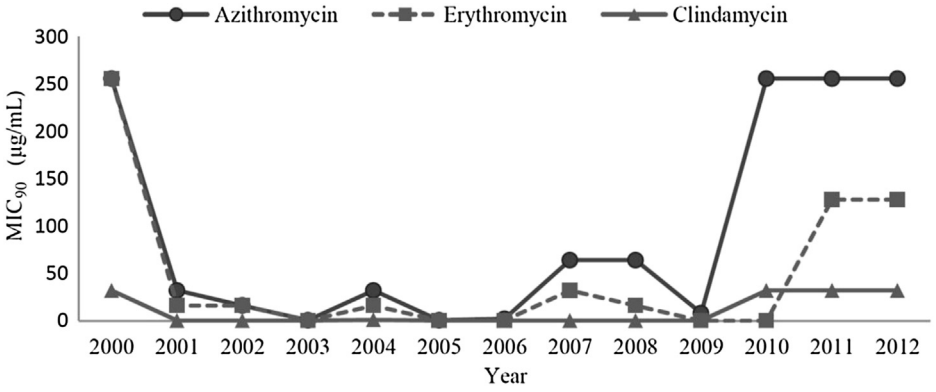

Figure 2. Annual trend of MIC_{90} (μg/mL) for azithromycin, erythromycin, and clindamycin during 2000–2012.

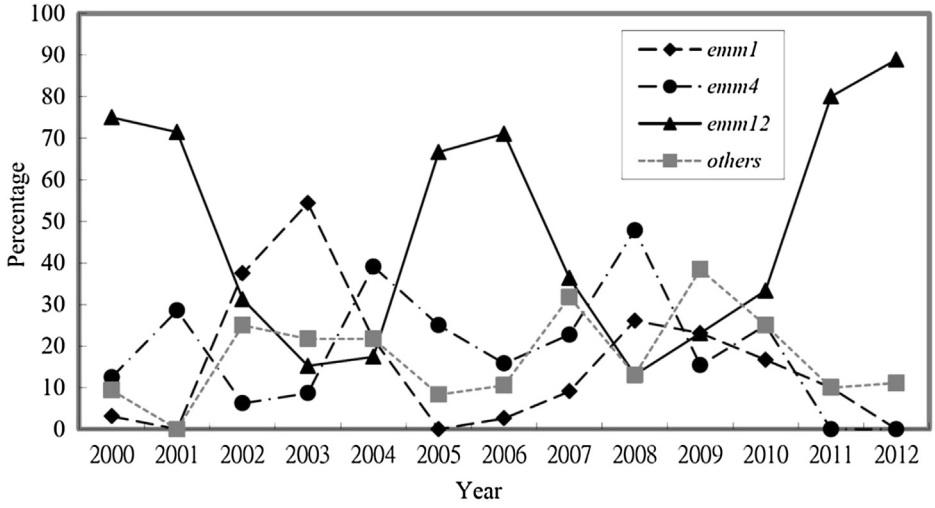

Figure 3. Annual trends in the distribution of *emm* types of group A streptococci during 2000—2012.

Spain, high resistance rates to erythromycin (32.8%) were found between 1994 and 2006, and the resistance rate to clindamycin and tetracycline was 6.5% and 6.8%, respectively.[17] In China, the resistance rates of clarithromycin, erythromycin, and azithromycin were 98.1%, 97.6%, and 97.2%, respectively. High clindamycin and tetracycline resistance rates (97.2% and 94.0%, respectively) were also found.[18]

However, in some areas of Europe and South America, the erythromycin resistance rates of GAS were low. In Norway, the erythromycin resistance rate was 2.7% within three separate periods from 1993 to 2002.[19] The erythromycin resistance rate was 2.6% during 2006—2009 in Germany. Low azithromycin and clindamycin resistance rates were also found (1.4% and 0.9%, respectively).[20] One study in Brazil showed that none of the 130 isolates collected from symptomatic children were resistant to erythromycin, azithromycin, or clindamycin.[21]

Although resistance rates to macrolide were high worldwide, the rates decreased in some countries gradually under antimicrobial stewardship program. In France, the resistance rate to erythromycin was high (22.4%) between 2002 and 2003 and as previously mentioned, it decreased to 12% between 2005 and 2006. It further declined to 3.2% in children with acute pharyngitis between 2009 and 2011 due to decreased macrolide consumption.[22] In Belgium, a large study showed decreased macrolide resistance from 13.5% in 1999 to 3.3% in 2006.[23] It was related to decreased consumption of macrolides, lincosamides, streptogramin B, and tetracycline.

In 2001, the SMART (Surveillance from Multicenter Antimicrobial Resistance in Taiwan) program collected isolates from nine hospitals in different parts of Taiwan, and the non-susceptibility rate to erythromycin was 78% (54% were intermediate, and 24% were resistant), and the nonsusceptibility rate to clindamycin was 5%.[24] In the current study, the erythromycin resistance rate were 53—57% in 2000 and 2001.

However, the resistance rate to erythromycin in Taiwan has decreased since 2002. Hsueh et al[5] demonstrated decreased erythromycin resistance in GAS, at 46% in 1999 to 17% in 2003 from three major hospitals in Taiwan, and the relationship was observed between decreased erythromycin consumption and decreased resistance rate. The decline in erythromycin use was due to Taiwan governmental policy beginning in February 2001 to deny reimbursement through the National Health Insurance (NHI) system for the costs of antibiotics for the treatment of acute upper respiratory tract infection without evidence of bacterial involvement.

Through implementation of regulations of antibiotics use, there was 33% total reduction of antimicrobials from 29.7 defined daily doses (DDDs) in 1999 to 19.8 DDDs in 2001 in ambulatory patients, and the use of erythromycin declined from 0.447 per 1000 inhabitants per day in 2000 to 0.129 DDDs per 1000 inhabitants per day in 2003.[25,26]

Our previous study in southern Taiwan showed a reduction in resistance rates among children, from 77% during 1997—1999 to 15% during 2002—2004.[27] Lo et al[28] showed decreased erythromycin resistance rates, from 40.7% during 1999—2002 to 24.7% during 2003—2005 in northern Taiwan. They also demonstrated low resistance rates (8%) in children with scarlet fever and pharyngitis during 2000—2011.[29] Another study showed a 6.8% erythromycin resistance rate from skin and soft tissue infections, whereas *emm*106 and *emm*11 were predominant.[30] The current study showed a significant decreased resistance rate to erythromycin and azithromycin since 2002. However, the resistance to macrolide has increased again since 2007.

Methylation of a ribosomal target and active efflux of erythromycin are the two leading factors involved in the resistance of streptococci to macrolides.[31] The predominant erythromycin-resistant mechanism of GAS strains varies in different geographic areas. Surveillance studies clearly showed *mef*A-mediated resistance prevailed in Mexico (95%) and Spain (89.5%), whereas the *erm*B predominates in China (90.3%) and France (69.4%), and *erm*TR predominated in United States (56%).[15,17,18,32] In the current study, *mef*A remained the major mechanism in southern Taiwan between 2000 and 2008, but *erm*B-contained isolates dominated in 2011 and 2012. Whether the evolution change is due to the selecting pressure for

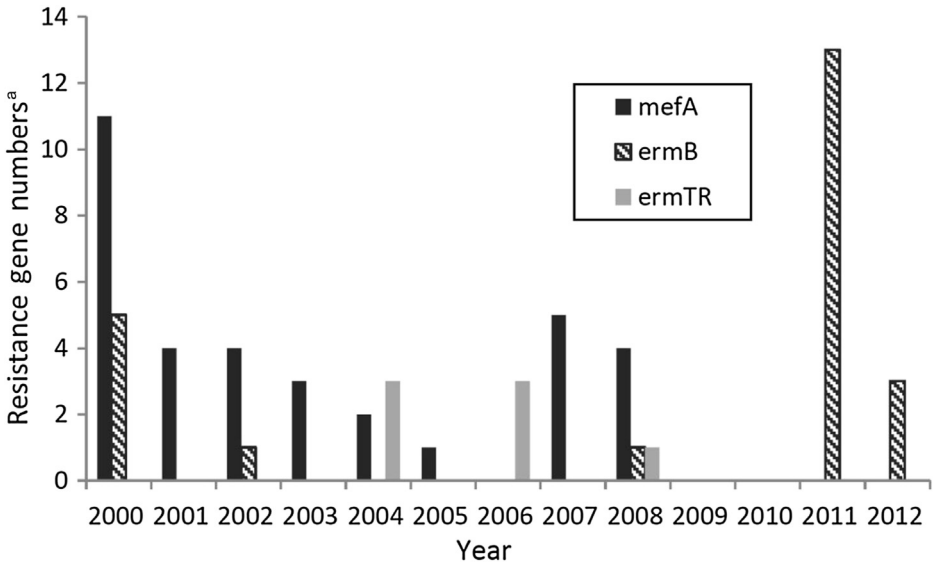

Figure 4. The distribution of erythromycin resistance genes among erythromycin-resistant strains of group A streptococci isolated from children during 2000—2012. [a] Resistance genes were not found in two erythromycin resistant isolates. Two resistance genes were detected in a single isolate.

resistance or environment influences requires additional study.

Epidemiological investigations suggested the spread of some *emm* strains was responsible for the increasing erythromycin resistance among GAS in several geographic areas. Martin et al[3] identified the spread of erythromycin-resistant *emm*6 strains in Pittsburgh. A few *emm*28 clones were associated with increasing resistance in French since 2002.[15] In the current study, various *emm* types were evident among erythromycin-resistant strains. *emm*4 and *emm*12, mainly originated from nasopharynx, were the most prevalent genotypes. *emm*12 strains were mainly detected in 2001—2002, 2005—2006, and 2011—2012, and *emm*4 strains in 2004 and 2008. Most *emm*12 isolates were obtained from patients with upper respiratory tract infection. Higher macrolide resistance rates of *emm*12 isolates may be related to overuse of macrolide in these patients. A PFGE study of nine erythromycin-resistant *emm*12 isolates randomly collected in 2011 and in 2012 showed almost the same PFGE among the isolates. The single clone of erythromycin resistant *emm*12 strain may be predominant in the community. However, there was no obvious outbreak of GAS infection during the recent 2 years.

In conclusion, not only a steady decline of erythromycin susceptibility but also the decreasing azithromycin and clindamycin susceptibility of the GAS strains was observed in southern Taiwan. This trend might be due to a complex interplay of different contributions, including possible increasing antibiotics consumption in the community, or the clonal spread of erythromycin-resistant strains. *mef*A and *erm*B contained strains remained the major mechanism of erythromycin resistance in this area. *erm*B and *erm*TR

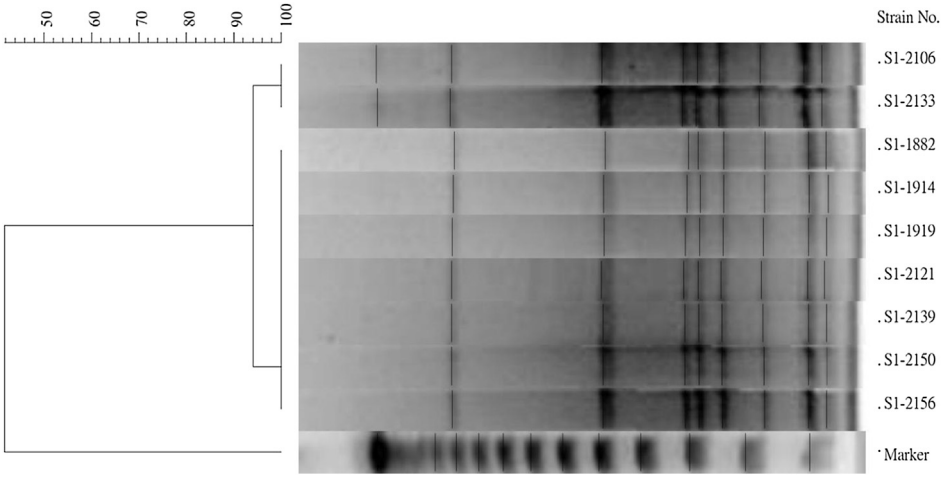

Figure 5. Pulsed-field gel electrophoresis patterns of *Sma*I restricted chromosomal DNA of *Streptococcus pyogenes emm*12 strains. A dendrogram was generated with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

contained strains were more frequent to have cross-resistance to more than two classes of antimicrobial agents. Whether the changes of antibiotics resistance and the molecular characterization among GAS isolates represents a trend or is just a temporal variation needs further surveillance.

Conflicts of interest

The authors declare that they have no financial or nonfinancial conflicts of interest related to the subject matter or materials discussed in the manuscript.

Acknowledgments

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