Molecular epidemiology, antimicrobial susceptibility, and characterization of macrolide-resistant *Streptococcus pyogenes* in Japan

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Original Article

1. Introduction

*Group A streptococci* (GAS) are known to cause a wide variety of human illnesses, including pharyngitis, impetigo, mastoiditis, and systemic infections, some of which can be life-threatening, such as sepsis, necrotizing fasciitis, septic arthritis, and toxic shock syndrome. In particular, for children, GAS infections are an important cause of morbidity and mortality worldwide [1,2].

Usually, penicillin is the first choice agent for the treatment of GAS infections. For severe infections, a combination of high-dose penicillin and clindamycin is recommended, while for patients with penicillin allergy, macrolide drugs are recommended as the first-line therapy. However, a high proportion of macrolide-resistant GAS (MRGAS) has been reported in many countries that have not introduced restrictions to macrolide use. In fact, macrolide resistance proportions have recently reached 32.8% in Spain, 40% in Belgium, 98.4% in China, and 22.8% in Greece [3–6]. The proportion of macrolide resistance of GAS is also reportedly high in Japan at 30%–40% [7].

The main macrolide resistance mechanisms of GAS are modification of the target site and efflux of macrolide drugs. Several genes, including *ermB*, *ermA* subtype TR (*ermTR*), *mefa*, and *msrD*, are associated with macrolide resistance [8,9]. *ermB* and *ermTR* encode 23S rRNA methylases which mediate target site modification, resulting in antibiotic resistance. These genes lead to resistance to macrolides, lincosamide, and streptogramin B by reducing the binding ability of these drugs (MLSb phenotype) [9]. *mefa* and *msrD* encode the transmembrane- and ATP-binding domains of pump that efflux C14 and C15 macrolides out of the cell. The *mefa* and *msrD* genes lead to resistance only to macrolide drugs (M phenotype) [8].

Among the various virulence factors of GAS that contribute to successful host invasion, the cell surface M protein plays a key role in GAS resistance to phagocytosis. The hypervariable 5′ region of the M protein, which is encoded by *emn*, is further classified by *emn* sequence typing [10]. Some studies reported that certain *emn*...
types were associated with severe infection, while others were associated with antimicrobial resistance. In addition, emm typing can vary among regions. Hence, the emm type serves as a useful marker that is often associated with predominant pathogenic strains [11].

This study aimed to determine the features of MRGAS isolates collected from children with pharyngotonsillitis in the southwestern areas of Japan over the past 3 years via emm typing, MLST, and PFGE. The findings of the present study were compared to those of a previous Japanese investigation [12] to further elucidate the mechanism of macrolide resistance of GAS in Japan.

2. Material and methods

2.1. Bacterial isolates

Between 2011 and 2013, a total of 299 GAS isolates were submitted for characterization to the Department of Pediatrics and Child Health of Kurume University of Medicine (Kurume, Japan) from four clinics (Shindo children’s clinic, Nagai children’s clinic, Ikewaza children’s clinic, and Tsumura clinic) and two general hospitals (Kurume University Hospital and St. Mary’s Hospital) in the southwestern areas of Japan. These strains were reconstituted from frozen stocks and propagated on sheep blood agar plates at 37 °C. Identification of Streptococcus pyogenes was confirmed by colony morphology, β-hemolysis on blood agar, the bacitracin test, the BinaxNOW® Strep A test (Alere Medical Co., Ltd., Chiba, Japan), and 16S rRNA polymerase chain reaction (PCR) analysis [8]. A total of 124 erythromycin-resistant isolates collected from patients with pharyngotonsillitis was selected for analysis (see the Antimicrobial susceptibility test section).

2.2. Antimicrobial susceptibility test

Minimum inhibitory concentrations (MICs) were determined using the broth dilution method performed in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). The reference strain Streptococcus pneumoniae ATCC 49619 was included as a control. GAS strains were tested against 6 antibiotics: penicillin G (Meiji Seika Pharma Co., Ltd., Tokyo, Japan), amoxicillin (Sigma–Aldrich Co., LLC, Tokyo, Japan), erythromycin (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), azithromycin (Pfizer Japan Inc., Tokyo, Japan), and clindamycin (Pfizer Japan Inc.). Susceptibility results were categorized according to the CLSI criteria [13]. The erythromycin-resistant (MIC ≥ 1 μg/ml) isolates were then selected as the study population.

2.3. Determination of macrolide-resistant phenotypes

The clindamycin-susceptible and erythromycin-resistant isolates were classified as phenotype M using the disk diffusion susceptibility test, which was performed in accordance with the CLSI recommendations [13]. Among the erythromycin-resistant strains, resistant phenotype patterns were classified as clindamycin-susceptible (M phenotype), -resistant (constitutive phenotype, cMLSb), or -inducible (inducible phenotype, iMLSb) [14].

2.4. Detection of erythromycin-resistant genes

All erythromycin-resistant isolates were screened by PCR for the erythromycin-resistance genes ermA, ermB, mefA, and msrD. PCR assays were performed according to previously described conditions for each individual primer pair [8,15,16].

2.5. T-serotype and emm type (emm/T types)

The T-serotype was identified using a slide agglutination test with type-specific antisera (DENKA SEIKEN Co., Ltd, Tokyo, Japan). emm sequencing was performed in accordance with the protocol of the CDC International Streptococcal Reference Laboratory (http://www.cdc.gov/streplab/M-ProteinGene-typing.html).

2.6. PFGE analysis

PFGE analysis was performed as previously described, with slight modifications [17]. In brief, chromosomal DNA was digested overnight at 30 °C with the restriction enzyme SgrAI (New England Biolabs Japan Inc., Tokyo, Japan). The electrophoresis conditions were 22 h with 0.5–40 s switch time ramp at a 120° angle and 6 V/cm using a Chief Mapper system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The DNA bands were stained with ethidium bromide and photographed. The interpretation of PFGE patterns was based on criteria described by Tenover et al. [18]. SgrAI profiles were coded alphabetically, and for closely related pulsotypes (differences in 2 or 3 bands), a number was added. PFGE profiles were analyzed using Quantity One® software version 4.6.3 (Bio-Rad Laboratories, Inc.), employing the unweighted pair group method with arithmetic mean with the Dice coefficient and a position tolerance of 1% [19].

2.7. MLST analysis

All macrolide-resistant isolates, which included nearly every emm type and PFGE cluster, were assessed by MLST in accordance with the protocol on the MLST website. The primers of 7 housekeeping genes (i.e., gki, gtr, muri, mutS, recP, xpt, and yiqL) were based on information from the MLST website [20]. The allele and sequence type were assigned using the MLST websites.

2.8. Ethical statement

An ethical approval or patients’ consent was not required since the study only includes microbiological samples sent to Department of Pediatrics and Child Health, Kurume University School of Medicine on an anonymized basis and did not involve human subjects or material, and patients could not be identified.

3. Results

3.1. Antimicrobial susceptibility

A total of 299 GAS isolates were collected between 2011 and 2013, which included 84, 149, and 66 isolates collected in 2011, 2012, and 2013, respectively. All 299 GAS isolates showed susceptibility to clindamycin. All macrolide-resistant isolates, which included nearly every emm type and PFGE cluster, were assessed by MLST in accordance with the protocol on the MLST website. The primers of 7 housekeeping genes (i.e., gki, gtr, muri, mutS, recP, xpt, and yiqL) were based on information from the MLST website [20]. The allele and sequence type were assigned using the MLST websites.
3.4. T-serotype and emm type (emm/T types)

Relationships between macrolide-resistant genes and erm were observed in this study. The predominant erm type was 0.125 g/ml and that of clindamycin was 128 g/ml (Table 3). No resistance was found to penicillin G and amoxicillin. Among the macrolide-resistant isolates, 76 (61.3%) possessed mefA and 46 (37.1%) possessed ermB. All the isolates possessing mefA also possessed msrD. No isolate possessed both mefA and ermB, and no strain possessed ermTR. In this study, no resistance genes were found in 2 isolates. The MIC50 values for mefA/msrD and erm were 16 and ≥128 μg/ml, respectively. MIC for each of the 45 isolates (97.8%) positive for ermB was ≥128 μg/ml. All strains that possessed ermB were classified as phenotype cMLSB (Table 2).

3.3. Detection of erythromycin-resistant genes

Among the macrolide-resistant isolates, 76 (61.3%) possessed mefA and 46 (37.1%) possessed ermB. All the isolates possessing mefA also possessed msrD. No isolate possessed both mefA and ermB, and no strain possessed ermTR. In this study, no resistance genes were found in 2 isolates. The MIC50 values for mefA/msrD and erm were 16 and ≥128 μg/ml, respectively. MIC for each of the 45 isolates (97.8%) positive for ermB was ≥128 μg/ml. All strains that possessed ermB were classified as phenotype cMLSB (Table 2).

3.2. Determination of macrolide resistance phenotype

Of the 124 erythromycin-resistant isolates, 76 were classified as phenotype M and 48 were classified as phenotype cMLSβ. There were no i MLSβ isolates in this study. For all the M phenotype isolates, MIC50 of erythromycin was 16 μg/ml and that of clindamycin was 0.125 μg/ml. For all the cMLSβ phenotype isolates, MIC50 of erythromycin was 128 μg/ml and that of clindamycin was 128 μg/ml (Table 2).

3.4. T-serotype and emm type (emm/T types)

Ten emm/T types were detected among all macrolide-resistant isolates (Table 3). The predominant emm/T types were emm11/T1 (57 isolates, 46.0%), followed by emm12/T12 (46 isolates, 37.1%), emm28/T28 (7 isolates, 5.6%), emm170/T25 (7 isolates, 5.6%), emm75/T4 (2 isolates, 1.6%), emm75/T4 (1 isolate, 0.8%), emm89/T (1 isolate, 0.8%), emm70/T5 (3 isolates, 2.4%), emm41/T4 (1 isolate, 0.8%), and emm12/NT (1 isolate, 0.8%). Of these emm/T types, macrolide resistance was particularly high for the emm12/T12 type with MIC50 for erythromycin of ≥128 μg/ml. The relationships between macrolide-resistant genes and emm/T types are shown in Table 3. ermB was detected in types emm1 and emm28. The mefA/msrD complex was detected in types emm1, emm12, emm4, emm75, and emm170.

3.5. MLST, emm typing, and PFGE

All macrolide-resistant isolates were subjected to MLST, which revealed the presence of 7 different sequence types: ST28, ST36, ST38, ST49, ST52, ST467, and ST646. Finally, 8 emm/MLST types were observed in this study population. The predominant emm1/MLST types were emm11/ST28 (57 isolates, 46.0%), followed by emm12/ST467 (24 isolates, 19.4%), emm12/ST36 (23 isolates, 18.5%), emm170/ST49 (8 isolates, 6.5%), emm28/ST52 (7 isolates, 5.6%), emm75/ST49 (3 isolates, 2.4%), emm89/ST646 (1 isolate, 0.8%), and emm41/ST38 (1 isolate, 0.8%) (Table 3).

All 124 isolates were subjected to PFGE using the restriction enzyme SgrAl. All the 124 SgrAl-digested macrolide-resistant isolates were assigned to 12 pulsotypes: A (57 isolates: 46.0%), B (24 isolates: 19.3%), C (11 isolates: 8.9%), D (9 isolates: 7.3%), E (7 isolates: 5.6%), F (5 isolates: 4.0%), G (5 isolates: 4.0%), H (2 isolates: 1.6%), I (1 isolate: 0.8%), J (1 isolate: 0.8%), K (1 isolate: 0.8%), and L (1 isolate: 0.8%).

PFGE, emm typing, and MLST results showed that most GAS isolates in the same PFGE pulseotype were the same emm and MLST types. In the pulsotype C group, there were 2 emm types: emm75 and emm170. The most predominant pulsotype was A/emm11/ST28 (57 strains: 46.0%), which possessed the mefA/msrD complex. The next most common pulsotype was B/emm12/ST467 (24 strains, 19.3%), which possessed ermB. The relationships between the PFGE pulseotype and emm/ST types are shown in Fig. 1.

There is no statistical difference against a certain emm/ST/PFGE type to macrolide-resistant proportion by year. The statistical analysis was performed according to the chi-square test.

4. Discussion

The prevalence of MRGAS was approximately <10% before 2000 [21], but it has gradually increased each year since then, and recent studies have reported that the prevalence of MRGAS is 30%–40% in Japan [7]. However, details regarding macrolide-resistant genes in Japan are insufficient. In this study, we found no isolates that possessed msrD genes. All but 2 isolates had mefA or ermB. There were no isolates which possessed mefA and ermB together. None of isolate possessed ermTR.

Table 1

<table>
<thead>
<tr>
<th>Agent</th>
<th>2011–2013 (n = 299) (μg/ml)</th>
<th>% resistance, by year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC50</td>
<td>MIC90</td>
</tr>
<tr>
<td>Erythromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.063–&gt;128</td>
<td>0.25</td>
<td>≥128</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.063–&gt;128</td>
<td>0.25</td>
<td>≥128</td>
</tr>
<tr>
<td>Azithromycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.063–&gt;128</td>
<td>0.25</td>
<td>≥128</td>
</tr>
<tr>
<td>Clindamycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤0.063–&gt;128</td>
<td>0.125</td>
<td>≥128</td>
</tr>
</tbody>
</table>

No resistance was found to penicillin G and amoxicillin.

Table 2

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. of isolates</th>
<th>Antimicrobial agent (μg/ml)</th>
<th>Erythromycin-resistant genes (no. of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC50</td>
<td>MIC90</td>
</tr>
<tr>
<td>M</td>
<td>76</td>
<td>EM</td>
<td>8–32</td>
</tr>
<tr>
<td>cMLSβ</td>
<td>48</td>
<td>EM</td>
<td>≤0.063–1</td>
</tr>
<tr>
<td>iMLSβ</td>
<td>0</td>
<td>EM</td>
<td>≤0.063–1</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>EM</td>
<td>≤0.063–&gt;128</td>
</tr>
</tbody>
</table>

The isolates of 76 strains possessed both the mefA genes and msrD genes. In this study, we found no isolates that possessed ermTR genes. All but 2 isolates had mefA or ermB. There were no isolates which possessed mefA and ermB together. None of isolate possessed ermTR.
mechanisms and characteristics of these isolates are lacking in these past reports. The purpose of this study was to survey the genetic diversity of pharyngeal GAS isolates by T typing, emm typing, MLST, and PFGE to identify factors related to the high proportion of macrolide resistance of GAS in Japan and to further elucidate the epidemiology of MRGAS.

The proportion of macrolide resistance varies by country. For example, among Asian countries, the incidence of macrolide resistance in Korea and Taiwan has decreased [22,23], but it has remained relatively high in China [4,24]. In European countries, the incidence of macrolide resistance has reportedly decreased in Germany and France [25]. The results of the present study showed that the proportion of MRGAS remained at >40%. Of all macrolide-resistant isolates included in this study, 61.3% were classified as phenotype M and 38.7% as phenotype cMLSB. The proportions of these resistance phenotypes were similar to those observed within other East Asian countries.

The GAS Surveillance Study Group in Japan reported that the most prevalent emm type of MRGAS was emm1, followed by emm12 and emm28. Each of these 3 emm types had high proportions of macrolide resistance (64.3%–87.2%) [26]. In our study, the most prevalent emm types of the MRGAS were emm1, emm12, and

<table>
<thead>
<tr>
<th>emm type</th>
<th>T type</th>
<th>MLST type</th>
<th>No. of isolates</th>
<th>Genes (no. of isolates)</th>
<th>MIC50 of EM (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>emm1</td>
<td>T1</td>
<td>ST28</td>
<td>57</td>
<td>0</td>
<td>57 16</td>
</tr>
<tr>
<td>emm4</td>
<td>T4</td>
<td>ST38</td>
<td>1</td>
<td>0</td>
<td>1 8</td>
</tr>
<tr>
<td>emm12</td>
<td>T12</td>
<td>ST36/ST467</td>
<td>46</td>
<td>7</td>
<td>7 128</td>
</tr>
<tr>
<td>emm12</td>
<td>NT</td>
<td>ST467</td>
<td>1</td>
<td>0</td>
<td>1 128</td>
</tr>
<tr>
<td>emm28</td>
<td>T28</td>
<td>ST52</td>
<td>7</td>
<td>0</td>
<td>7 128</td>
</tr>
<tr>
<td>emm75</td>
<td>T4</td>
<td>ST49</td>
<td>1</td>
<td>0</td>
<td>1 16</td>
</tr>
<tr>
<td>emm75</td>
<td>T25</td>
<td>ST49</td>
<td>2</td>
<td>2</td>
<td>2 16</td>
</tr>
<tr>
<td>emm89</td>
<td>TB3264</td>
<td>ST646</td>
<td>1</td>
<td>0</td>
<td>0 128</td>
</tr>
<tr>
<td>emm170</td>
<td>T25</td>
<td>ST49</td>
<td>7</td>
<td>7</td>
<td>7 16</td>
</tr>
<tr>
<td>emm170</td>
<td>T5/27/44</td>
<td>ST49</td>
<td>1</td>
<td>1</td>
<td>1 8</td>
</tr>
</tbody>
</table>

Table 3
Distribution of emm/T types/MLST, genes, and erythromycin-resistant isolates (n = 124).

Fig. 1. Dendrogram and PFGE patterns of SgrAI-digested chromosomal DNA, and association with phenotype, emm type, sequence type, and isolation year in erythromycin-resistant GAS (n = 124). DNA size standards (lambda ladder; 50–1000 kb). SgrAI-digested isolates generated 12 pulsotypes (A–L) and closely related pulsotypes (differences in 2 or 3 bands) were assigned to each PFGE clusters (A, E, F). NT: nontypeable.
emm28. The emm12 and emm4 types are reportedly the most common in various countries, while emm4 strains were the most predominant among MRGAS, particularly in Europe [25,28].

Of the M phenotype isolates, there were 5 emm/ST types (emm1/ST28, emm75/ST49, emm170/ST49, emm12/ST36, and emm4/ST38) and 4 PFGE pulsortypes (A, C, E, and I). emm1/ST28 was the most prevalent in our study. The domestic surveillance results showed that the predominant prevalent in our study. The domestic surveillance results showed that the predominant among MRGAS were ST28 and ST661 [26]. In our study, emm1/ST28 was also the most predominant type, suggesting that this type was the most prevalent genotype among pharyngotonsillitis cases over the past several years in Japan. With regard to the PFGE band patterns, emm1/ST28 had only 1 pulsortype (pulsotype A), which revealed that the most prevalent MRGAS type. emm1/ST28 had the same genetic homology and macrolide resistance was mainly related to the prevalence of this type clones in Japan. Of the macrolide-resistant proportions by year, the highest proportion of macrolide-resistance was observed in 2011. In this study we could not find the relationship of a certain emm/ST/PFGE type with macrolide-resistant proportion. Therefore, it could be suggested that there was no outbreak by a certain emm/ST/PFGE type in MRGAS in the southwestern areas of Japan. Although individual emm types were associated with multiple PFGE patterns, emm1/ST28/ST661 (emm170) shared the same PFGE type. The emm sequence st1815 (emm170) was likely generated by homologous excision between tandem emm and emm sequences in an emm75 parental strain, as suggested in the emm sequence database (http://www.cdc.gov/streplab/types-emm103-124.html).

Among the isolates with the cMLS9 phenotype, there were 4 emm/ST types (emm12/ST467, emm12/ST36, emm28/ST52, and emm89/ST646) and 9 PFGE pulsortypes (B, D, E, F, G, H, K, and L). emm12/ST467 was the most prevalent in our study. emm12 type ST465 or ST36 was the most prevalent in a previous Japanese investigation between April and October 2012 [26], whereas emm12/ST467 (19.4%, 24/124 strains) rather than emm12/ST36 (18.5%, 23/124 strains) was the most prevalent in our study. We suggested that one of the reasons for the difference in these two studies was the period of investigation. Their study period was short; therefore, their investigation may not exactly express the prevalence situation of emm/ST type, and our investigation could not be compared the previous abovementioned investigation. ST465 and ST467 are single-locus variants of ST36. The results of these two studies indicate that the clonal variant emm12/ST36 was widespread in Japan.

Studies conducted in Europe and East Asia reported that emm12 strains were the most predominant among MRGAS [27,30]. With regard to the PFGE band patterns, emm12/ST467 was clustered into 1 PFGE pattern (pulsotype B) and emm12/ST36 was clustered into 4 patterns (pulsotypes D, E, G, and H), which had significantly different patterns from pulsotype B. To our knowledge, this is the first report to reveal that emm12/ST467 is one of the predominant types of MRGAS in Japan. Our findings suggest that emm12/ST467 is a novel MRGAS in East Asian countries, including Japan.

Several studies have reported a statistically significant association between macrolide resistance and emm28 [29,32], and most emm28 strains had the emmB gene [31]. Although few articles have reported the distribution of emm28 in other Asian countries, in our study, 7 of 124 strains were identified as emm28/ST52, and all possessed the emmB gene. This finding suggests that the characteristics of emm28 are the same worldwide. The prevalence of emm28 increased throughout this study period and, thus, may be associated with an increase in MRGAS detection.

Several studies have reported correlations between the increase in MRGAS prevalence and macrolide use [33–35]. In response, some countries have started restricting the use of some antibiotics, including macrolides and decreased the proportion of MRGAS [29,33]. These reports showed antimicrobial stewardship including restriction of macrolide drugs lead decreasing proportion of MRGAS. However, some anti-inflammatory effects have been reported for a 14-member macrolide drug used [36]. Macrolide drugs are often used inadequately for the treatment of respiratory infections such as those caused by influenza virus in Japan. This phenomenon is in contrast to the global situation, which may be one of the reasons why the proportion of macrolide resistance has remained greater than 40%.

In this study we investigated the typing among only macrolide-resistant strains, therefore we could not indicate the relationship between macrolide-resistant proportions and typing such as emm typing and MLST among whole strains included macrolide-susceptible strains. In further investigations, we need to analyze the typing of all provided isolates, and investigate for more wide area to determine the feature of GAS isolates exactly.

In summary, we found high proportions of MRGAS and the prevalence of 2 predominant genotypes (emm12/ST28 and emm12/ST467) in Japan.

Conflict of interest

None.

Acknowledgments

We thank Kensuke Nagai and Shigeru Ikezawa for their support and useful comments.

References


