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Original Article

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

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ABSTRACT

Here we report the molecular epidemiology of macrolide-resistant *Streptococcus pyogenes* (group A streptococci, GAS) isolated from children with pharyngotonsillitis between 2011 and 2013 in Japan. In 299 isolates, 124 (41.5%) isolates were macrolide-resistant. We characterized the isolates by *emm* typing, multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). Of 299 isolates, 124 (41.5%) were macrolide-resistant isolates, 76 (61.3%) possessed *mef*A and 46 (37.1%) possessed *erm*B. All 76 isolates with *mef*A possessed *msr*D. There were no isolates possessed *erm*TR in this study. Eight *emm*/MLST types were observed. The predominant type was *emm*1/ST28 (57 isolates, 46.0%), which possessed the *mef*A/*msr*D complex, presenting as the M phenotype. The second most predominant type was *emm*12/ST467, which possessed *erm*B, presenting as the $cMLS_B$ phenotype. Of the $cMLS_B$ phenotype isolates, types *emm*28/ST52 and *emm*12/ST36 had multiple genetic backgrounds. We found high proportions of macrolide-resistant GAS in the southwestern areas of Japan.

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 *erm*B, *erm*A subtype TR (*erm*TR), *mef*A, and *msr*D, are associated with macrolide resistance [8,9]. *erm*B and *erm*TR 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 (MLS_B phenotype) [9]. *mef*A and *msr*D encode the transmembrane- and ATP-binding domains of pump that efflux C14 and C15 macrolides out of the cell. The *mef*A and *msr*D 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 *emm*, is further classified by *emm* sequence typing [10]. Some studies reported that certain *emm*

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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, Ikezawa 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), clarithromycin (Taisho 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 $\geq$ 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, $cMLS_B$), or -inducible (inducible phenotype, $iMLS_B$) [14].

2.4. Detection of erythromycin-resistant genes

All erythromycin-resistant isolates were screened by PCR for the erythromycin-resistance genes *erm*B, *erm*A, *mef*A, and *msr*D. 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 *Sgr*AI (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 CHEF 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]. *Sgr*AI 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 penicillin G and amoxicillin. Of the 299 GAS isolates, 124 (41.5%) were erythromycin-resistant, including 44 (52.4%), 54 (36.2%), and 26 (39.4%) collected in 2011, 2012, and 2013, respectively. The proportions of resistance to clarithromycin, azithromycin, and clindamycin were 41.5%, 42.8%, and 16.4%, respectively. A total of 101 isolates (33.8%) were highly resistant to erythromycin (MIC $\geq$ 16 μg/ml). Of the 299 GAS isolates, $MIC_{50/90}$ values for erythromycin were 0.25/$\geq$128 μg/ml (Table 1). None of the erythromycin-resistant isolates were susceptible to clarithromycin or azithromycin, whereas 73 erythromycin-resistant isolates were susceptible to clindamycin.

Table 1
Antimicrobial susceptibilities of GAS isolates from 2011 to 2013.

Agent	2011–2013 (n = 299) (μg/ml)			% resistance, by year		
	Range	MIC_{50}	MIC_{90}	2011 (n = 84)	2012 (n = 149)	2013 (n = 66)
Erythromycin	≤0.063–≥128	0.25	≥128	52.4	36.2	39.4
Clarithromycin	≤0.063–≥128	0.25	≥128	52.4	36.2	39.4
Azithromycin	≤0.063–≥128	0.5	≥128	54.8	36.2	42.4
Clindamycin	≤0.063–≥128	0.125	≥128	17.9	14.8	19.7

No resistance was found to penicillin G and amoxicillin.

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 $cMLS_B$. There were no $iMLS_B$ isolates in this study. For all the M phenotype isolates, MIC_{50} of erythromycin was 16 μg/ml and that of clindamycin was 0.125 μg/ml. For all the $cMLS_B$ phenotype isolates, MIC_{50} of erythromycin was 128 μg/ml and that of clindamycin was 128 μg/ml (Table 2).

3.3. Detection of erythromycin-resistant genes

Among the macrolide-resistant isolates, 76 (61.3%) possessed *mef*A and 46 (37.1%) possessed *erm*B. All the isolates possessing *mef*A also possessed *msr*D. No isolate possessed both *mef*A and *erm*B, and no strain possessed *erm*TR. In this study, no resistance genes were found in 2 isolates. The MIC_{90} values for *mef*A/*msr*D and *erm*B were 16 and ≥128 μg/ml, respectively. MIC for each of the 45 isolates (97.8%) positive for *erm*B was ≥128 μg/ml. All strains that possessed *erm*B were classified as phenotype $cMLS_B$ (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 *emm*1/T1 (57 isolates, 46.0%), followed by *emm*12/T12 (46 isolates, 37.1%), *emm*28/T28 (7 isolates, 5.6%), *emm*170/T25 (7 isolates, 5.6%), *emm*75/T25 (2 isolates, 1.6%), *emm*75/T4 (1 isolate, 0.8%), *emm*89/T B3264 (1 isolate, 0.8%), *emm*170/T5/27/44 (1 isolate, 0.8%), *emm*4/T4 (1 isolate, 0.8%), and *emm*12/NT (1 isolate, 0.8%). Of these *emm*/T types, macrolide resistance was particularly high for the *emm*12/T12 type with MIC_{50} for erythromycin of ≥128 μg/ml. The relationships between macrolide-resistant genes and *emm*/T types are shown in Table 3. *erm*B was detected in types *emm*12 and *emm*28. The *mef*A/*msr*D complex was detected in types *emm*1, *emm*12, *emm*4, *emm*75, and *emm*170.

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 *emm*/MLST types were *emm*1/ST28 (57 isolates, 46.0%), followed by *emm*12/ST467 (24 isolates, 19.4%), *emm*12/ST36 (23 isolates, 18.5%), *emm*170/ST49 (8 isolates, 6.5%), *emm*28/ST52 (7 isolates, 5.6%), *emm*75/ST49 (3 isolates, 2.4%), *emm*89/ST646 (1 isolate, 0.8%), and *emm*4/ST38 (1 isolate, 0.8%) (Table 3).

All 124 isolates were subjected to PFGE using the restriction enzyme *Sgr*AI. All the 124 *Sgr*AI-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 pulsotype were the same *emm* and MLST types. In the pulsotype C group, there were 2 *emm* types: *emm*75 and *emm*170. The most predominant pulsotype was A/*emm*1/ST28 (57 strains: 46.0%), which possessed the *mef*A/*msr*D complex. The next most common pulsotype was B/*emm*12/ST467 (24 strains, 19.3%), which possessed *erm*B. The relationships between the PFGE pulsotype 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

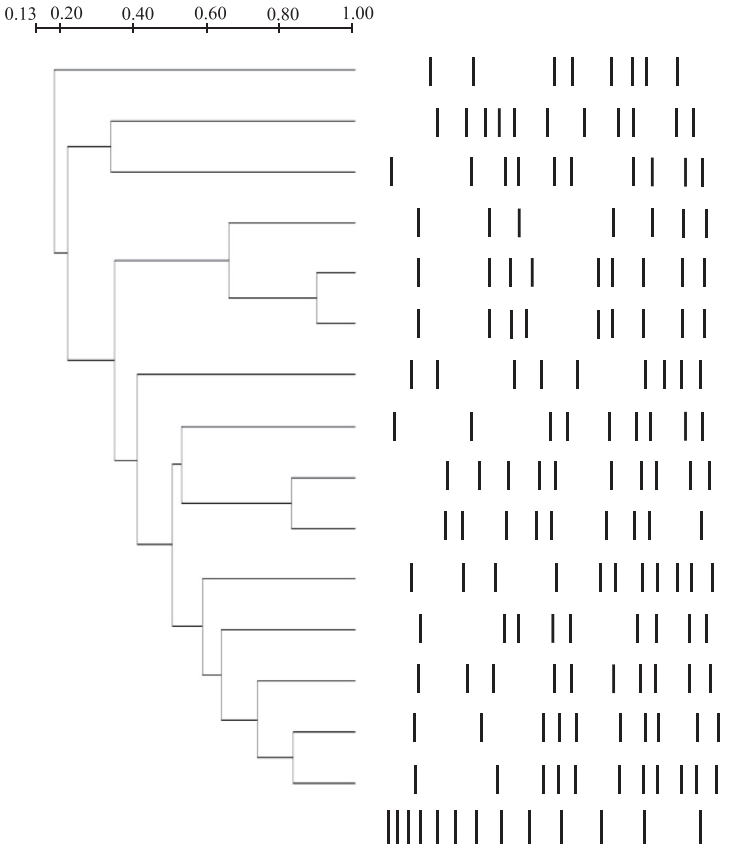
Table 2
Distribution of MICs, phenotype, and genotypes of erythromycin-resistant genes (n = 124).

Phenotype	No. of isolates		Antimicrobial agent (μg/ml)			Erythromycin-resistant genes (no. of isolates)								
			Range	MIC_{50}	MIC_{90}	*mef*A			*erm*B			*msr*D		
						2011	2012	2013	2011	2012	2013	2011	2012	2013
M	76	EM	8–32	16	32	31	32	13	0	0	0	31	32	13
		CLDM	≤0.063–1	0.125	0.25									
cMLSB	48	EM	64–≥128	≥128	≥128	0	0	0	11	22	13	0	0	0
		CLDM	64–≥128	≥128	≥128									
iMLSB	0	EM	–	ND	ND	0	0	0	0	0	0	0	0	0
		CLDM	–	ND	ND									
						31	32	13	11	22	13	31	32	13
Total	124	EM	8–≥128	16	≥128	76			46			76		
		CLDM	≤0.063–≥128	≥128	≥128									

The isolates of 76 strains possessed both the *mef*A genes and *msr*D genes. In this study, we found no isolates that possessed *erm*TR genes. All but 2 isolates had *mef*A or *erm*B. There were no isolates which possessed *mef* A and *erm*B together. None of isolate possessed *erm*TR.

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

emm type	T type	MLST type	No. of isolates	Genes (no. of isolates)			MIC_{50} of EM (μg/ml)
				*mef*A	*erm*B	*msr*D	
*emm*1	T1	ST28	57	57	0	57	16
*emm*4	T4	ST38	1	1	0	1	8
*emm*12	T12	ST36/ST467	46	7	38	7	≥128
*emm*12	NT	ST467	1	0	1	0	≥128
*emm*28	T28	ST52	7	0	7	0	≥128
*emm*75	T4	ST49	1	1	0	1	16
*emm*75	T25	ST49	2	2	0	2	16
*emm*89	TB3264	ST646	1	0	0	0	≥128
*emm*170	T25	ST49	7	7	0	7	16
*emm*170	T5/27/44	ST49	1	1	0	1	8
Total			124	76	46	76	



PFGE cluster	*emm* type	MLST	Pheno-type	No. of isolates 2011	2012	2013	No. of isolates
B	12	467	M				0
			$cMLS_B$	9	13	2	24
K	28	52	M				0
			$cMLS_B$			1	1
J	28	52	M				0
			$cMLS_B$		1		1
C	75/170	49	M	5	5	1	11
			$cMLS_B$				0
A1	1	28	M	19	16	11	46
			$cMLS_B$				0
A2	1	28	M	5	6		11
			$cMLS_B$				0
I	4	38	M		1		1
			$cMLS_B$				0
G	12	36	M				0
			$cMLS_B$	1	4		5
F1	28	52	M				0
			$cMLS_B$		1	2	3
F2	28	52	M				0
			$cMLS_B$			2	2
H	12	36	M				0
			$cMLS_B$	2			2
L	89	646	M				0
			$cMLS_B$	1			1
D	12	36	M				0
			$cMLS_B$		3	6	9
E1	12	36	M		4	1	5
			$cMLS_B$				0
E2	12	36	M				0
			$cMLS_B$	2			2
			M	29	32	13	74
			$cMLS_B$	15	22	13	50
			Total	44	54	26	124

Fig. 1. Dendrogram and PFGE patterns of *Sgr*AI-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). *Sgr*AI-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.

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 $cMLS_B$. 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 *emm*1, followed by *emm*12 and *emm*28. 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 *emm*1, *emm*12, and

*emm*28. The *emm*12 and *emm*4 types are reportedly the most common in various countries, while *emm*4 strains were the most predominant among MRGAS, particularly in Europe [25,28].

Of the M phenotype isolates, there were 5 *emm*/ST types (*emm*1/ST28, *emm*75/ST49, *emm*170/ST49, *emm*12/ST36, and *emm*4/ST38) and 4 PFGE pulsotypes (A, C, E, and I). *emm*1/ST28 was the most prevalent in our study. The domestic surveillance results showed that the predominant *emm*1 types among the MRGAS were ST28 and ST661 [26]. In our study, *emm*1/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, *emm*1/ST28 had only 1 pulsotype (pulsotype A), which revealed that the most prevalent MRGAS type. *emm*1/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 of MRGAS in the southwestern areas of Japan.

Although individual *emm* types were associated with multiple PFGE patterns, *emm*75 and *emm* st1815 (*emm*170) shared the same PFGE type. The *emm* sequence st1815 (*emm*170) was likely generated by homologous excision between tandem *emm* and *enn* sequences in an *emm*75 parental strain, as suggested in the *emm* sequence database (http://www.cdc.gov/streplab/types-emm103-124.html).

Among the isolates with the $cMLS_B$ phenotype, there were 4 *emm*/ST types (*emm*12/ST467, *emm*12/ST36, *emm*28/ST52, and *emm*89/ST646) and 9 PFGE pulsotypes (B, D, E, F, G, H, J, K, and L). *emm*12/ST467 was the most prevalent in our study. *emm*12 type ST465 or ST36 was the most prevalent in a previous Japanese investigation between April and October 2012 [26], whereas *emm*12/ST467 (19.4%, 24/124 strains) rather than *emm*12/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 *emm*12/ST36 was widespread in Japan.

Studies conducted in Europe and East Asia reported that *emm*12 strains were the most predominant among MRGAS [27,30]. With regard to the PFGE band patterns, *emm*12/ST467 was clustered into 1 PFGE pattern (pulsotype B) and *emm*12/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 *emm*12/ST467 is one of the predominant types of MRGAS in Japan. Our findings suggest that *emm*12/ST467 is a novel MRGAS in East Asian countries, including Japan.

Several studies have reported a statistically significant association between macrolide resistance and *emm*28 [29,32], and most *emm*28 strains had the *erm*B gene [31]. Although few articles have reported the distribution of *emm*28 in other Asian countries, in our study, 7 of 124 strains were identified as *emm*28/ST52, and all possessed the *erm*B gene. This finding suggests that the characteristics of *emm*28 are the same worldwide. The prevalence of *emm*28 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 analysis 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 (*emm*1/ST28 and *emm*12/ST467) in Japan.

Conflict of interest

None.

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