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Extremely Low Prevalence of Erythromycin-Resistant *Streptococcus pyogenes* Isolates and Their Molecular Characteristics by M Protein Gene and Multilocus Sequence Typing Methods

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Abstract

Background: Group A *streptococci* (GAS) are notorious bacteria causing a wide variety of clinical manifestations ranging from mild, acute streptococcal pharyngitis to chronic non-suppurative diseases and immunological sequelae. They are further complicated by the global rise on the emergence of macrolide resistance among these bacteria in which several M protein gene (*emm*) and sequence types are associated with invasive diseases.
Objectives: The current study aimed at determining the erythromycin resistance patterns and molecular characteristics of GAS clinical strains by *emm* and multilocus sequence typing (MLST) methods.
Methods: Thirty-five GAS clinical isolates were subjected to antibiotic susceptibility testing by disk diffusion method. The minimum inhibitory concentration (MIC) of erythromycin against GAS by E-test was determined. Clinical and laboratory standards institute (CLSI) guideline was used for the interpretation of results. Detection of *ermA*, *ermB*, and *mefA* genes by polymerase chain reaction (PCR) was performed and *emm* typing was done by amplification and sequencing of *emm* genes per standard protocol. Allele and sequence type (ST) of GAS were obtained using the *S. pyogenes* MLST database.
Results: All the isolates were sensitive to erythromycin, penicillin, clindamycin, chloramphenicol, and vancomycin (100%). Resistance to tetracycline was 54.3%. The *mefA* gene was found in one erythromycin susceptible isolate. No other erythromycin resistance genes were detected in the isolates. Twenty different *emm* types were found and the most frequent *emm* types/subtypes detected were *emm*1, *emm*18.21, *emm*28.5, *emm*97.4, and *emm*102.2 (each 8.6%). However, no new *emm* type was detected. A total of 15 sequence types (STs), eight clonal clusters (CCs), and eight singletons were identified among 21 representative isolates. Three isolates exhibited CC1 (ST28/*emm*1).
Conclusions: High susceptibility of GAS isolates against erythromycin could be due to low antibiotic selective pressure in Malaysian clinical settings. High diversity of *emm* and ST types revealed the heterogenic nature of the strains circulating in Malaysian hospitals. Continuous epidemiological monitoring by molecular typing methods is warranted to improve the management strategies of GAS infections in future.

Keywords: Streptococcus, Macrolides, Multilocus Sequence Typing, Malaysia

1. Background

Streptococcus pyogenes is a notoriously pathogenic bacterium causing a wide spectrum of diseases ranging from mild pharyngitis to severe life-threatening conditions such as streptococcal toxic shock syndrome and septicemia. Group A streptococci (GAS) disease and its sequelae such as acute rheumatic fever (ARF), rheumatic heart disease (RHD), and acute post-streptococcal glomerulonephritis (APSGN) have contributed to significant morbidity and mortality worldwide (1). Globally, 663,000 new cases and 163,000 deaths are reported each year due to invasive GAS diseases (1). Moreover, the global emergence of macrolide-resistance strains possesses a major concern for patients with β-lactam allergy (2).

The current macrolide resistance problem is mediated by horizontal transfer of macrolide resistance genes such as *ermA*, *ermB*, and *mefA* as well as the spread of resis-

tant GAS clones (3). The *ermA* and *ermB* genes, which encode proteins for ribosomal methylation as well as *mefA* gene, which encodes protein for macrolide efflux mechanism, are responsible for antibiotic resistance in GAS (4). Macrolide-resistant *S. pyogenes* isolates are documented from various studies in some Asian countries and these resistant strains can be transmitted among neighboring countries (5, 6).

The M protein is encoded by *emm* in the 5' end of the hypervariable region. For the past centuries, typing of M protein was performed using specific M-typing antisera (7). Nonetheless, the reagents that were not widely available and difficulties in performing the test became the major drawbacks for its usage. In addition, there are reports of GAS strains that could not be typed by the specific antisera; thus, making it difficult to determine the distribution of M-types (7, 8). The *emm* typing is the analysis of the sequence of the hypervariable portion of *emm* gene, which is used as an alternative of M-typing using antisera. It is now considered as the gold standard since it can characterize the virulence associated M protein, which is an important epidemiological marker of *S. pyogenes* infections (9). Interestingly, certain *emm* types are associated with invasive diseases as well as antibiotic resistance (10).

Apart from *emm* typing, better characterization of the GAS genetic lineages are proposed by the use of multilocus sequence typing (MLST) (11). MLST is a typing method that utilizes nucleotide sequence determination and targets seven neutral house-keeping genes of bacteria. The method is used to determine the relationship amongst bacteria of the same species (4). It was found very useful in the characterization and monitoring of antibiotic resistance and bacterial pathogenicity as well as the purpose of epidemiological studies since the data can be compared across the globe (12). To the authors' knowledge, data on the erythromycin susceptibility pattern, *emm* type and sequence type of GAS, is still lacking among local strains in Malaysia.

2. Objectives

The current study aimed at determining the prevalence of erythromycin resistance using disk diffusion and E-test methods, detecting erythromycin resistance genes (*ermA*, *ermB* and *mefA*) by polymerase chain reaction (PCR), and characterizing the genetic profiles of GAS by *emm* and MLST typing methods.

3. Methods

3.1. Ethics Statement

The protocol of the study was approved by the institutional ethical committee (UPM/TNCPI/RMC/JKEUPM/1.4.18.1/F1).

3.2. Sampling and Identification

A total of 35 non-repetitive *S. pyogenes* species were isolated from patients in two Malaysian hospitals from August 2013 to September 2014. Clinical samples were collected from skin pus (n = 18) and blood (n = 8), followed by tissue (n = 6) and throat swabs (n = 2) (obtained from patients with gangrene and tonsillitis, respectively), and non-healing diabetic wound swab (n = 1). Invasive and non-invasive GAS isolates were categorized based on the source of isolation (13). *Streptococcus pyogenes* was identified by PYR test (Oxoid, United Kingdom), bacitracin susceptibility (Oxoid, United Kingdom), latex agglutination (Oxoid, United Kingdom), and 16SrRNA sequencing.

3.3. Antibacterial Susceptibility Testing

Antimicrobial susceptibility testing was done by disk diffusion method on Mueller-Hinton agar (Difco, United States) supplemented with 5% sheep blood and incubated in 5% CO_2 for 24 hours at 37°C according to CLSI guideline (14). The tested antibiotics were as follows: penicillin G (10 μg), erythromycin (15 μg), clindamycin (2 μg), chloramphenicol (30 μg), tetracycline (30 μg), and vancomycin (30 μg) (Oxoid, United Kingdom). The minimum inhibitory concentration (MIC) of erythromycin was determined by E-test and interpreted based on CLSI guidelines (14). While establishing the PCR protocols, one erythromycin-susceptible isolate consistently demonstrated the presence of *mefA* gene in triplicate. Thus, detection of erythromycin resistance genes (*ermA*, *ermB* and *mefA*) was conducted in all the isolates, according to the protocol previously described (15). GOTaq Green Master Mix (Promega, USA) and Biometra thermocycler (Biorad®, Germany) were used for PCR amplification. PCR products were analyzed by gel electrophoresis in a 2% (w/v) agarose gel (Invitrogen, USA). *Streptococcus pneumoniae* ATCC 49619 was used as a negative control, while *Staphylococcus aureus* BAA ATCC 977, *S. pneumoniae* ATCC 700677, and *S. pneumoniae* ATCC 700676 were used as positive controls for *ermA*, *ermB*, and *mefA*, respectively.

3.4. Sequencing of emm Gene

Sequencing of the 5' region of the *emm* gene was done in accordance with the protocol provided by the centre for disease control and prevention (CDC). Lysates of all the

isolates were prepared and amplification was carried out by PCR using Biometra thermocycler (BioRad®, Germany). Purification and sequencing were performed by first base laboratories (1st Base laboratory Sdn Bhd, Malaysia). Sequences were edited using Bioedit software version 7.0 and compared with reference sequences using the BLAST algorithm. The *emm* pattern was referred to the established data based on the different types of *emm* obtained in the current study (16). Twenty-one representative isolates were chosen for MLST, based on the predominant *emm* types with the exclusion of similar isolation sites. Amplification of seven housekeeping genes and sequencing were determined as previously described (17). The MLST sequences and clonal relatedness represented as clonal clusters using eBURST clustering method were queried into the database at http://spyogenes.mlst.net. Isolates with the same *emm* type and sequence type (ST) were considered as the same clone.

4. Results

All isolates (100%) were susceptible to erythromycin, penicillin, clindamycin, chloramphenicol, and vancomycin (Table 1). However, 54.3% of GAS was resistant to tetracycline. Among the three types of resistance genes, only *mefA* gene was detected in one erythromycin-susceptible isolate (Figure 1). The sequence showed 99% identity to the GenBank sequence with the accession number of dbj|AB513667.1 (Figure 2). Twenty different *emm* types were identified, and *emm*1, *emm*18.21, *emm*28.5, *emm*97.4, and *emm*102.2 types/subtypes were the most frequently detected ones (8.6% for each), followed by 5.7% of *emm*63, *emm*71, *emm*76.4, *emm*89, and *emm*91 (Figure 3). No new *emm* types were identified. The *emm* pattern E (45.7%) was predominantly detected in the current study (Table 1). MLST analysis demonstrated eight predominant clonal clusters (CCs) and six singletons with diverse sequence types (STs) (Table 2). The predominant STs and CCs among the representative isolates were identified as follows: ST28/*emm*1 (CC1, 14.3%), ST60/*emm*102 (CC58, 9.5%) and ST473/*emm*28 (CC76, 9.5%), while each of these singletons (ST318/*emm*71 and ST402/*emm*18) was represented by 9.5% of the isolates (Table 2).

5. Discussion

Macrolide-resistant GAS raised a considerable concern among the clinician and scientist since macrolides are commonly used as a second-line alternative for treatment (2). In addition, various reports documented the emergence of macrolide-resistant GAS in some Asian countries

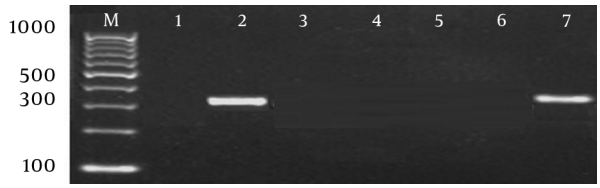

Figure 1. Gel electrophoresis image of *mefA* resistance gene; M: 100-bp ladder; lane 1: S. pneumoniae ATCC 49619 as negative control; lane 2: *S. pneumoniae* ATCC 700677 as positive control; lane 3 - 6: representative *S. pyogenes* isolates; lane 7: positive isolate with *mefA* gene (348 bp)

recently (6, 18). Ironically, all isolates (100%) were susceptible to erythromycin in the current study. High susceptibility rate (92.2%) was also observed among GAS isolates in Japan (19). More recently, an increasing trend of susceptibility towards erythromycin was reported from several countries such as Taiwan and Portugal (5, 20). These findings may be probably explained by the judicious use of erythromycin or other contributing factors (20, 21). Nonetheless, high resistance against erythromycin was reported from other countries. For instance, 15.4% and 7.0% of the isolates were also resistant to erythromycin in Brazil and Spain, respectively (22, 23). Differences in the prevalence of macrolide-resistant GAS are commonly related to geographical area, type of circulating strains or clones, and types of study population. In addition, *S. pyogenes* in the present study remained susceptible to penicillin and vancomycin as reported by others (3, 5, 24).

As for the clindamycin resistance, all GAS isolates in the current study were also susceptible to this antibiotic. It is well understood that clindamycin has immunemodulatory properties and is commonly used for the treatment of severe GAS infections (25). Surprisingly, a high resistance rate for tetracycline (54.3%) was observed in the present study. In contrast, only 3.5% of GAS isolates were resistant to tetracycline in Spain (23). However, the current study result was in agreement with the reports on global rise of tetracycline-resistant GAS (26, 27). With regard to the resistance gene, the presence of *mefA* in an erythromycin-susceptible isolate in the current study is not surprising. Similar observations were documented in a study by Brenciani et al. in which *tetM* gene was found in tetracycline-susceptible isolates (28). It was proposed that the resistance genes could be present in a silence form (28). Nonetheless, further study is warranted to evaluate the significance of this finding in future.

Twenty different *emm* types were identified in the current study. However, no new *emm* types were observed. A wide diversity of *emm* types was observed with no dominancy of a single *emm* type in the current study. Similar observation was reported in United Arab Emirates (29). Inter-

Table 1. Distribution of Antimicrobial Susceptibility Patterns, Source of Isolation, and *emm* Patterns of *Streptococcus pyogenes* Clinical Isolates[a]

Source	Antimicrobial Susceptibility Pattern												*emm* Pattern, n[b]
	P		E[c]		DA		TE		VA		Cl		
	S	R	S	R	S	R	S	R	S	R	S	R	
Pus	51.4	0	51.4	0	51.4	0	25.7	25.7	51.4	0	51.4	0	A - C (1), D (9), E (8)
Blood	22.9	0	22.9	0	22.9	0	5.7	17.1	22.9	0	22.9	0	A - C (3), D (3), E (2)
Tissue	17.1	0	17.1	0	17.1	0	8.5	8.5	17.1	0	17.1	0	A - C (2), E (4)
Wound	5.7	0	5.7	0	5.7	0	2.9	2.9	5.7	0	5.7	0	A-C (1), E (1)
Throat	2.9	0	2.9	0	2.9	0	2.9	0	2.9	0	2.9	0	E (1)
Total	100	0	100	0	100	0	45.7	54.3	100	0	100	0	

Abbreviations: C, chloramphenicol (30 μg); DA, clindamycin (2 μg); E, erythromycin (15 μg); n: frequency; P, penicillin G (10 μg); R, resistance; S, sensitive; TE, tetracycline (30 μg); VA, vancomycin (30 μg).
[a]Values are expressed as %.
[b]Described as pattern A - C: throat specialist; pattern D: skin specialist; pattern E: a generalist.
[c]Confirmed by E-test with MIC (susceptible < 0.25 μg/mL, intermediate 0.5 μg/mL, resistant > 1 μg/mL).

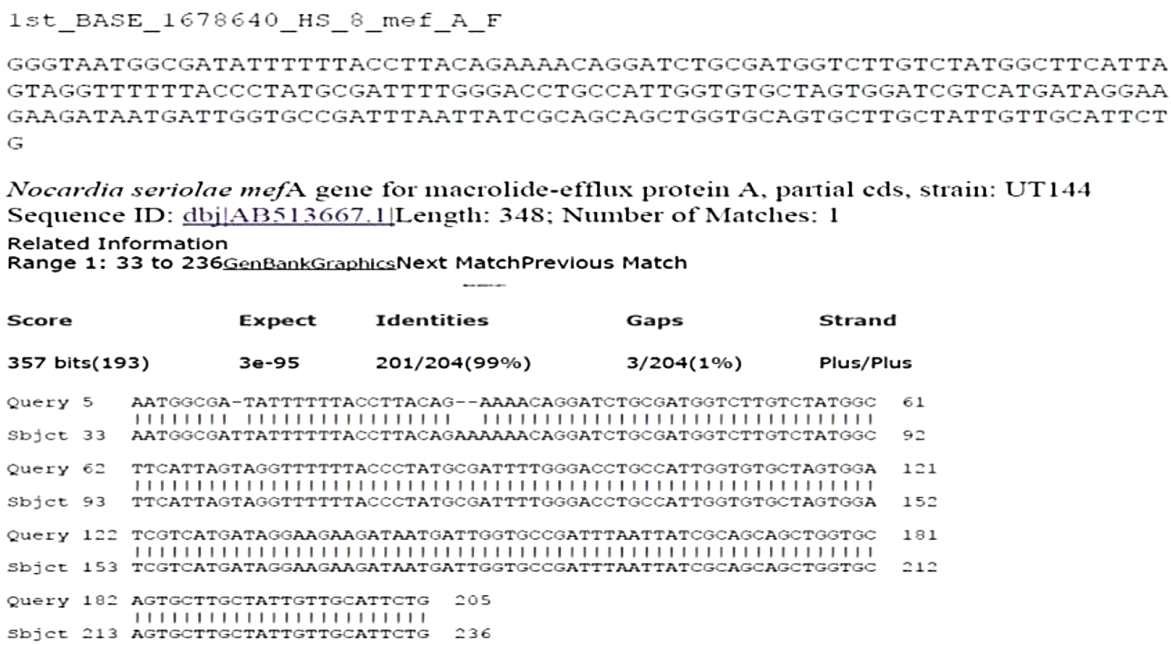

Figure 2. GenBank sequence result of *mefA* with 99% homology

estingly, erythromycin-susceptible GAS isolates with *emm* types of 1, 12, 81, and 89 found in the present study were also observed by others (11, 30). In contrast to current study findings, *emm* types of 12 and 89 were associated with erythromycin resistance in few studies (31, 32). It is well understood that the distribution of *emm* types may differ according to geographical region. It is proposed that the specific genetic marker (*emm* pattern) could be related to tissue preference. With regard to this, *emm* pattern E (45.7%) and *emm* patterns A-C (20.0%) were identified from both invasive and non-invasive sites in the present study. The *emm* pattern E has no predilection to any specific tissue sites (33). In contrast to the current study findings, *emm* patterns A - C are usually related to throat carriage and non-

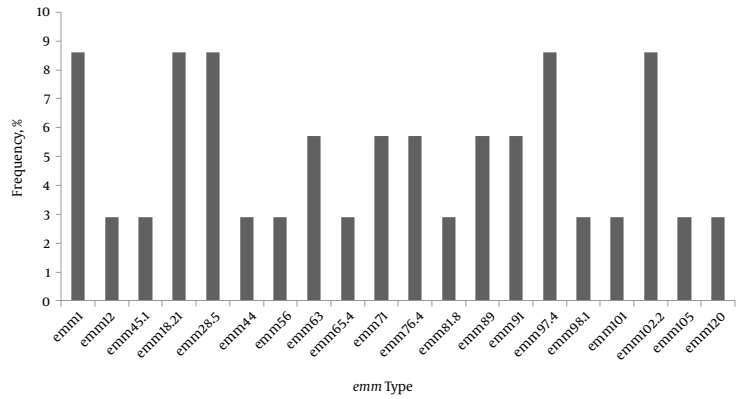

Figure 3. Distribution of *emm* types and subtypes among 35 *Streptococcus pyogenes* recovered from clinical samples

Table 2. Distribution of Housekeeping Allele Profiles, Sequence Types, Clonal Complexes, and *emm* Types Among Representative GAS Isolates

No of Isolate	*gki*	*gtr*	*murI*	*mutS*	*recP*	*xpt*	*yiqL*	ST	CC	*emm* Type
HS 1	4	3	4	4	4	2	4	28	CC1	1
HS 2	13	2	14	1	9	3	1	60	CC58	102
HS 4	4	6	4	4	52	31	2	300	Singleton	97
HS 5	42	3	2	2	2	2	1	599	CC83	76
HS 6	4	3	62	4	4	2	1	313	Singleton	97
HS 9	4	2	3	11	17	3	1	25	CC32	76
HS 10	4	3	4	4	4	2	4	28	CC1	1
HKL 2	16	2	8	3	1	13	3	101	CC2	89
HKL 3	2	2	2	3	2	3	2	5	CC26	91
HKL 5	38	24	2	37	2	3	1	426	Singleton	63
HKL 6	16	2	8	3	82	13	3	408	CC2	89
HKL 7	4	36	8	7	51	3	76	473	CC76	28
HKL 9	4	3	4	4	4	2	4	28	CC1	1
HKL11	69	2	2	7	1	3	12	402	Singleton	18
HKL12	69	2	2	7	1	3	12	402	Singleton	18
HKL14	2	10	35	7	2	57	1	318	Singleton	71
HKL17	13	2	14	1	9	3	1	60	CC58	102
HKL18	2	6	2	5	22	3	2	13	CC78	91
HKL19	4	36	8	7	51	3	76	473	CC76	28
HKL22	2	2	1	50	2	3	1	306	Singleton	63
HKL23	2	10	35	7	2	57	1	318	Singleton	71

Abbreviations: CC, clonal cluster; Gki, glucose kinase gene; gtr, glucose transporter protein gene; HKL, hospital Kuala Lumpur; HS, Hospital Serdang; murI, glutamate racemase gene; mutS, DNA mismatch repair protein gene; recP, transketolase gene; ST, sequence type; xpt, xanthine phosphoribosyl transferase gene; yiqL, acetyl-CoA acetyltransferase gene.

invasiveness (33). Whereas, 9 (75.0%) out of 12 *emm* pattern D (skin specialist) were identified from the skin pus.

The *emm* types of 1, 12, 18, 28, 44, 71, and 81 found in the current study are amongst 25 most common *emm* types causing overall disease in Asia (34). More interestingly, 4 *emm* types (n = 6; 42.9% of total invasive sites) comprising

*emm*1 (n = 1), *emm*12 (n = 1), *emm*18 (n = 3), and *emm*101 (n = 1) are usually linked to invasive disease in Asia (34). The crucial development of vaccine is based on the detection of predominant *emm* types among invasive GAS strains and the target population involved. To the authors' best knowledge, it is the first study on the distribution of *emm* type in Malaysia based on *emm* typing. Thus, this may give little insight for the development of vaccine in future.

MLST analysis demonstrated eight predominant CCs with diverse STs among 21 isolates in the current study. The predominant STs among the representative isolates were as follows (in descending order): ST28/*emm*1 (CC1, 14.3%) and ST60/*emm*102 (CC58, 9.5%), while each of these singletons (ST318/*emm*71 and ST402/*emm*18) were represented by 9.5% of the isolates. The occurrence of the globally acknowledged groups such as the ST28/*emm*1 (CC1) found in both hospitals in the study is of particular concern as it is linked to invasive diseases (34). The *emm*89 with different STs consisting of ST101 and ST408 both under CC2 were observed in 9.5% of the isolates. Some *emm* types represented by two isolates each portrayed widely divergent genetic backgrounds. Amongst them, *emm*63 (ST426 and ST306) both singletons, differed at 4 of the 7 housekeeping loci, *emm*91 represented by ST5 (CC26) and ST13 (CC78) showed differences at 5 of the 7 loci. Isolates with *emm* types of 1, 18, 28, 71, and 102 in the present study shared identical or highly similar allelic profiles, also observed in other studies (26, 35). The study has several limitations. Limited types of *emm* could be due to the small number of isolates in the present study. Thus, the predominant *emm* types could not be considered as the basis for vaccine development in Malaysia.

6. Conclusions

Group A *Streptococcus* isolates found in the current study displayed high susceptibility towards erythromycin, which could be due to low antibiotic selection pressure in Malaysian clinical settings. High genetic diversity of GAS strains are observed by *emm* and MLST typing methods. The presence of ST28/*emm*1 (CC1) among the GAS deserves special attention due to its invasive characteristics. Thus, continuous epidemiological monitoring by molecular typing methods is warranted in future.

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Footnotes

Authors' Contribution: Study concept and design: Rukman Awang Hamat, Hauwa Mohammed Kalgo, Azmiza Syawani Jasni; analysis and interpretation of data: Rukman Awang Hamat, Nurul, Siti Rohani, Siti Nur Adila; drafting: Hauwa Mohammed Kalgo; critical revision of the manuscript for important intellectual content: Rukman Awang Hamat.

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