ORIGINAL ARTICLE

Distribution of virulence genes and the molecular epidemiology of *Streptococcus pyogenes* clinical isolates by *emm* and multilocus sequence typing methods

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ABSTRACT

Background: *Streptococcus pyogenes* has a variety of virulence factors and the predominant invasive strains differ according to specific *emm* types and geographical orientation. Although *emm* typing is commonly used as the gold standard method for the molecular characterisation, multilocus sequence typing (MLST) has become an important tool for comparing the genetic profiles globally. This study aimed to screen selected virulence genes from invasive and non-invasive clinical samples and to characterise the molecular epidemiology by *emm* typing and MLST methods.

Materials and Methods: A total of 42 *S. pyogenes* isolates from invasive and non-invasive samples collected from two different tertiary hospitals were investigated for the distribution of virulence factors and their molecular epidemiology by *emm* and multilocus sequence typing methods. Detection of five virulence genes (*speA, speB, speJ, ssa* and *sdaB*) was performed using multiplex polymerase chain reaction (PCR) using the standard primers and established protocol. Phylogenetic tree branches were constructed from sequence analysis utilised by neighbour joining method generated from seven housekeeping genes using MEGA X software.

Results: Multiplex PCR analysis revealed that *sdaB/speF* (78.6%) and *speB* (61.9%) were the predominant virulence genes. Regardless of the type of invasiveness, diverse distribution of *emm* types/subtypes was noted which comprised of 27 different *emm* types/subtypes. The predominant *emm* types/subtypes were *emm*63 and *emm*18 with each gene accounted for 11.8% whereas 12% for each gene was noted for *emm*28, *emm*97.4 and *emm*91. The MLST revealed that the main sequence type (ST) in invasive samples was ST402 (17.7%) while ST473 and ST318 (12% for each ST) were the major types in non-invasive samples. Out of 18 virulotypes, Virulotype A (five genes, 55.6%) and Virulotype B (two genes, 27.8%) were the major virulotypes found in this study. Phylogenetic analysis indicated the presence of seven different clusters of *S. pyogenes*. Interestingly, Cluster VI showed that selected *emm*/ST types such as *emm*71/ST318 (n=2), *emm*70.1/ST318 (n=1), *emm*44/ST31 (n=1) and *emm*18/ST442 (n=1) have clustered within a common group (Virulotype A) for both hospitals studied.

Conclusion: The present study showed that group A streptococcci (GAS) are genetically diverse and possess virulence genes regardless of their invasiveness. Majority of the GAS exhibited no restricted pattern of virulotypes except for a few distinct clusters. Therefore, it can be concluded that virulotyping is partially useful for characterising a heterogeneous population of GAS in hospitals.

KEYWORDS:
Emm type; Multilocus sequence typing; Streptococcus pyogenes; virulence genes

INTRODUCTION

Group *A Streptococcus* (GAS) or better known as *Streptococcus pyogenes* possesses various virulence factors that are involved in severe life-threatening infections. Invasive *S. pyogenes* infections have contributed to high lethality rates ranging from 10 to 30%, resulting in more than 600,000 deaths worldwide.[1] Virulence factors such as superantigens (SAgs), adhesins, proteases and leukocidins play a major role in the pathogenesis of *S. pyogenes* diseases.[2] For instance, adhesins are groups of proteins which are responsible for the initial attachment of *S. pyogenes* to epithelial cells.[3]

In the presence of other virulence factors such as M protein and streptokinase, *speB* gene can contribute to GAS pathogenicity.[4] Interestingly, specific serotypes of highly virulent *S. pyogenes* strains are recognised to express more than one fibronectin-binding proteins. The M1 and M49 serotypes were found to produce a cellular surface fibronectin-binding protein (FbaA), which has a pivotal role in *S. pyogenes* invasion into deeper tissues.[5] Superantigens such as streptococcal pyrogenic exotoxins (SpeA, SpeC and SpeG to SpeM) and the streptococcal superantigen (SSA) have been shown to mediate massive uncontrolled inflammatory reactions by overstimulating the host inflammatory cells mainly T-cell lymphocytes in severe invasive *S. pyogenes* infections.[6] Some of these toxin genes are exclusively

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expressed in certain *S. pyogenes* serotypes. The M1 serotype that has a specific combination of *speA* and *smeZ* genes were found to increase the pathogenicity of this strain.[7]

To date, studies on the distribution of *S. pyogenes* serotypes and their virulence genes are limited in Malaysia.[8,9] Moreover, previous findings on *S. pyogenes* serotypes and virulence factors were characterised based on M-typing using specific antisera which is obsolete currently as the reagents are not widely available.[10] Besides, some of the *S. pyogenes* strains could not be typed by the antisera, thus limits the use of M-typing as the typing method of choice.[8,10] The *emm* typing method has now been accepted as the gold standard since M-protein is encoded by the 5' end of the hypervariable region of *emm* gene, and this gene can be sequenced for the typing purposes.[11] Meanwhile, *S. pyogenes* genetic lineages can be characterised and globally compared using the multilocus sequence typing (MLST) method.[12] This method utilises seven highly conserved house-keeping genes and different nucleotide sequences can be determined to characterise different sequence types.[12] Previous data showed that certain *emm* types are associated with different types of *S. pyogenes* disease manifestations and invasiveness and the epidemiology of *emm* types is geographically oriented.[13,14] Therefore, to better understand the epidemiology of *S. pyogenes* infections in Malaysia, this pilot study aimed to characterise the selected virulence genes of *S. pyogenes* from various clinical specimens via *emm* typing and MLST methods.

MATERIALS AND METHODS

The present study was conducted in 2018 by utilising the previous collection of 42 *S. pyogenes* isolates which were obtained from two different tertiary hospitals from the year 2014 to 2015. The distance between these two hospitals is approximately 30km apart. The isolates were stored at -70°C and re-identification of the isolates was performed using the Gram staining method, bacitracin susceptibility (Oxoid, Basingstoke, United Kingdom), PYR test (Oxoid, Basingstoke, United Kingdom), latex agglutination (Oxoid, Basingstoke, United Kingdom), and species-specific polymer chain reaction (PCR) method.[15] The isolates were collected from blood (n=10), pus (n=22), tissue (n=7), wound (n=2) and throat (n=1). The sources for the isolates were categorised into invasive and non-invasive samples based on Creti et al.[16] The approval to conduct the study was obtained from the Ethics Committee for Research involving Human Subjects of Universiti Putra Malaysia with the reference number of UPM/TNCPI/RMC/1.4.18.2.

Multiplex polymerase chain reaction for the detection of selected virulence genes

Fresh bacterial colonies growing on Columbia agar enriched with 5% of sheep blood (Isolab Sdn. Bhd, Shah Alam, Selangor, Malaysia) were used for DNA extraction using the HiYield Genomic DNA kit (Real Biotech Corporation, Taipei, Taiwan) in accordance to the manufacturer's instructions. DNA extracts were stored at -30°C for further use.

Determination of five virulence genes (*speA, speB, speJ, sdaB,* and *ssa*) was carried out using a multiplex PCR kit (Qiagen, Germantown, USA) using few sets of primers (Table I) according to the multiplex PCR protocol described in a previous study.[17] An aliquot of DNA template (1µL) was transferred into an Eppendorf tube containing 12.5µL Qiagen® Muµtiplex PCR master mix with HotStarTaq DNA polymerase (Qiagen, Germantown, USA), 0.5µL of each virulence gene primer pair and 4.5µL multiplex PCR buffer. DNA amplification was performed using a Bio-Rad thermal cycler (Bio-Rad Laboratories Ltd., Watford, Hertfordshire, UK) with an initial denaturation process at 95°C for 3 min, followed by 35 cycles (30s at 94°C, 90 s at 57.4°C, 90s at 72°C) and the final extension process at 72°C for 10min. The PCR products were analysed with gel electrophoresis using 2% agarose gel containing 1× TBE buffer and 1µL of gel stain (Nanogene Solutions Sdn Bhd, Batu Caves, Selangor, Malaysia). The gel was then viewed using a gel documenting system Alpha image TM 2200 (Alpha Innotech Cooperation, San Liandro, USA). The multiplex PCR products of five virulence genes were sequenced and blasted with the GenBank sequences for the similarity index.

The emm typing of S. pyogenes

The *emm* typing was performed according to the recommended protocol by the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/ncidod/biotech/strep/protocols.htm). Bacterial DNA of all the isolates were prepared and PCR technique was used for DNA amplification using a Bio-Rad thermal cycler (Bio-Rad, California, UK). A set of forward and reverse primers were used as follows: 5'-TATT(CG)GCTTAGAAAATTAA-3' and 5'-GCAAGTTCTTCAGCTTGTTT-3', respectively. The PCR cycling conditions used were as follows: 94°C for 15 s, 46°C for 30 s, and 72°C for 75 s for the first 10 cycles, and then 94°C for 15 s, 46°C for 30 s and 72°C for 75 s (with a 10s increment for each of the subsequent 19 cycles). DNA purification and sequencing were performed by the 1st Base Laboratory Sdn. Bhd., Seri Kembangan, Malaysia. Received sequences were then edited using Bioedit software version 7.0 (https://bioedit.software.informer.com/7.0/) and compared with reference sequences using the BLAST algorithm.

Multilocus sequence typing (MLST) and phylogenetic study

The MLST was performed by sequencing seven housekeeping genes (*gki, gtr, murI, mutS, recP, xpt,* and *yqiL*) for all the isolates according to the established protocol with slight modifications.[18] A total of 4µL DNA template was mixed with 25µL of green master mix (Vivantis Technologies Sdn. Bhd., Subang Jaya, Selangor, Malaysia), 1.0µL of each housekeeping primer pair, and 19µL of PCR buffer. The PCR amplification was performed using a Bio-Rad thermal cycler (Bio-Rad, California, UK). An initial denaturation process was conducted at 95°C for 5 min, followed by 35 cycles (45 s at 55°C, 90 s at 72°C) and the final extension process at 72°C for 1 min. The sequencing analysis of DNA was conducted by MytacG Sdn. Bhd, Kajang, Selangor (Malaysia) and the data was submitted to the NCBI website for determination of gene similarity percentage using the BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence alignment was then analysed using a MEGA X software (https://www.megasoftware.net/home). In each locus, the different sequence was assigned by a distinct allele number, generating a seven-integer allelic profile for each isolate. Isolates with similar allelic profiles were assigned to the similar sequence type (ST). A complete database of alleles,

Table I: List of primer used for selected virulence genes in the present study

Virulence Gene	Forward primer (5'→3')	Reverse primer (3'→5')	Amplicon size (bp)
ssa	CGGAGGTGTTACTGAGCAC	GGTGCGGGCATCATATCGTA	274bp
speA	CCCCTCCGTAGATACATGCAC	ACACGACCAAGATTCAAGCCT	305bp
speJ	CTTTCATGGGTACGGAAGTGT	GCTCTCGACCTCAGAATCAACT	196bp
speB	AGACGGAAGAAGCCGTCAGA	TCAAAGCAGGTGCACGAAGC	952bp
sdaB	TATAGCGCATGCCGCCTTTT	TGATGGCGCAAGCAAGTACC	440bp

Table II: The distribution of five selected virulence genes according to the type of samples

Type of samples	*sdaB* n (%)	*speB* n (%)	*speJ* n (%)	*speA* n (%)	*ssa* n (%)
Invasive (n=17)	15 (45.5)	8 (30.8)	8 (36.4)	7 (35.0)	2 (11.1)
Non-invasive (n=25)	18 (54.5)	18 (69.2)	14 (63.6)	13 (65.0)	16 (88.9)
Total=42	33 (78.6)	26 (61.9)	22 (52.4)	20 (47.6)	18 (42.9)

Table III: Distribution of *emm* types, subtypes and sequence type (STs) according to the type of samples in this study

Type of samples	*emm* typing / subtyping (%)	Sequence type (%)
Invasive (n=17)	*emm63 (11.8), emm18 (11.8), emm1 (5.9), emm102.2 (5.9), emm76.5 (5.9), emm98.1 (5.9), emm12 (5.9), emm101 (5.9), emm81.8 (5.9), emm18.21 (5.9), emm15.1 (5.9), emm105 (5.9), emm86.2 (5.9), emm100.4 (5.9), emm89.28 (5.9)*	ST402 (17.7), ST28 (11.8), ST205(11.8), ST13 (5.9), ST60 (5.9), ST36 (5.9), ST25 (5.9), ST156 (5.9), ST442 (5.9), ST306 (5.9), ST147 (5.9), ST114 (5.9), ST549 (5.9), ST89 (5.9)
Non-invasive (n=25)	*emm28 (12), emm97.4 (12), emm91 (12), emm1 (8), emm71 (8), emm89 (8), emm102.2 (4), , emm76.5 (4), emm120 (4), emm63 (4), emm44 (4), emm6.1 (4), emm56 (4), emm70.1 (4), emm17.2 (4), emm57 (4)*	ST473 (12), ST318 (12), ST13 (8), ST313 (8), ST101 ST101 (8), ST55 (8), ST28 (4), ST60 (4), ST599 (4), ST168 (4), ST5 (4), ST426 (4), ST408 (4), ST31 (4), ST83 (4), ST300 (4)

allele sequences, and STs was obtained from the www.mlst.net website. Phylogenetic analysis was performed using the MEGA X software to produce distance-based dendrograms[19] and clustering of each isolate was performed using the MEGA X Sequence Alignment Editor to align the nucleotide sequences. Subsequently, phylogenetic tree branches were constructed using the aligned sequence obtained from the MUSCLE alignment in MEGA X software and information collected with the application of the neighbour-joining method using the MEGA X software.[20]

RESULTS

Among the 42 *S. pyogenes* isolates, the predominant virulence genes detected were as follows: *sdaB* (78.6%), *speB* (61.9%), *speJ* (52.4%), *speA* (47.6%) and *ssa* (42.9%). The distribution of five virulence genes in the invasive and non-invasive samples is shown in Table II. The gel electrophoresis patterns of multiplex PCR products are shown in Figure 1.

Table III shows the distribution of *S. pyogenes emm* types/subtypes according to the type of samples. In total, 27 different *emm* types/subtypes were detected in this study. No new *emm* types/subtypes were found. The predominant *emm* types/subtypes in invasive and non-invasive samples were *emm*63, *emm*18, *emm*28, *emm*97.4 and *emm*91 with 12% recorded for each gene. Meanwhile, application of MLST method indicated that the predominant sequence type (ST) in invasive samples was ST402 (17.7%) whereas ST473 and ST318 were the main ST in non-invasive samples with 12% recorded for each ST (Table III).

There were 18 different virulotypes detected, nevertheless, the main virulotypes were Virulotype A (five genes, 55.6%) and Virulotype B (two genes, 27.8%). This was followed by Virulotype C, D, E, F (16.7% for each); and Virulotype G, H, I (11.1% for each). Virulotype J to R represented 5.6% for each virulotype. The phylogenetic analysis revealed the prevalence of seven clusters of *S. pyogenes* in two different hospitals. Cluster V exhibited restricted patterns of two virulotypes which have three Virulotype A and two Virulotype L virulence genes. Meanwhile, a few *emm*/ST types shared only Virulotype A in Cluster VI. Figure 2 exhibits the phylogenetic tree of *S. pyogenes* strains inferred with the neighbour-joining method using concatenated sequences of seven housekeeping genes.

DISCUSSION

Streptococcus pyogenes is a human pathogen that is responsible for multiple infections globally. The pathogenic properties of *S. pyogenes* are often associated with the production of virulence factors such as superantigens, proteinases and adhesins. In particular, streptococcal erythrogenic exotoxins (SPEs) are involved in a massive inflammatory response and tissue destruction.[21] Thus, it is very important to determine the pathogenic potential of *S. pyogenes* strains and categorise them according to the MLST method for global comparison. Moreover, superantigen profiles of *S. pyogenes* strains are associated with specific *emm* types and their association differs across different countries.[22]

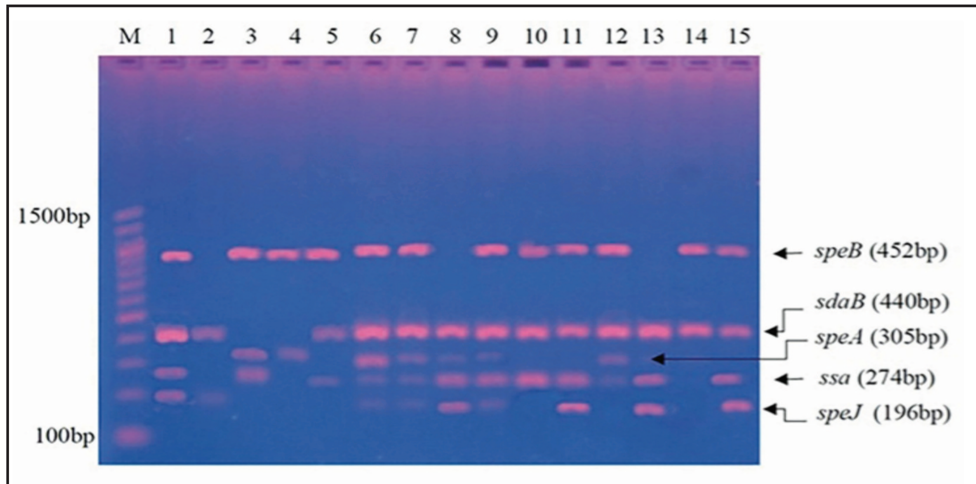

Fig. 1: Agarose gel electrophoresis patterns showing multiplex PCR amplification product for the *S. pyogenes* virulence genes. Lane M (Nanogene Solutions Sdn. Bhd., Batu Caves, Selangor, Malaysia): DNA molecular size marker (100bp). The specific virulence genes are labelled with arrows. Lane 1 to 10 represents isolates sampled from Hospital Serdang; Lane 11 to 15 represents isolates sampled from Hospital Kuala Lumpur.

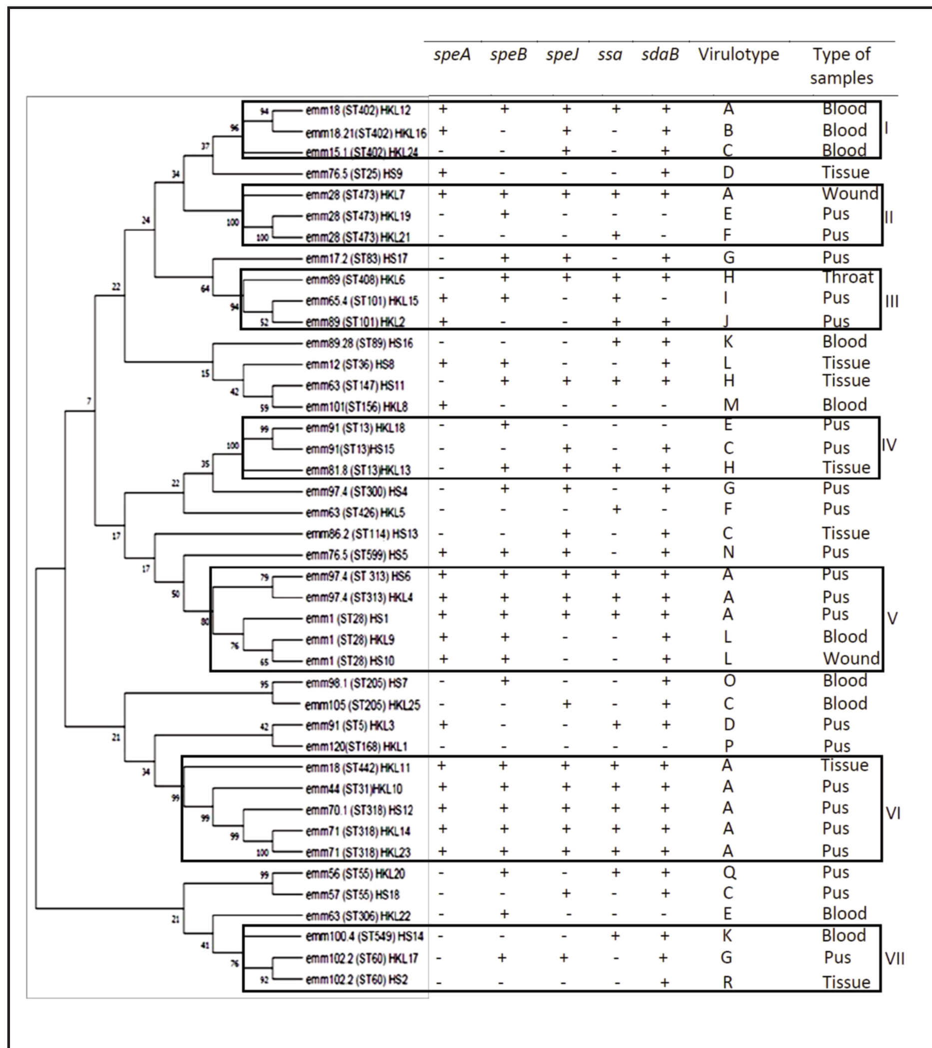

Fig. 2: Phylogenetic relationship among *S. pyogenes* strains inferred with the neighbor-joining method using concatenated sequences of seven housekeeping genes used for MLST. The taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Cluster V and VI showed *S.pyogenes* strains with two restricted virulotypes (A and L) and only Virulotype A, respectively in two different hospitals. HS=Hospital Serdang; HKL= Hospital Kuala Lumpur. Virulotype A=five genes; Virulotype D and N=four genes; Virulotype E, F, H, J, L and Q=three genes; Virulotype B, I, K and O=two genes; Virulotype C, G, M and R (one gene); Virulotype P=no gene detected.

In the present study, all *S. pyogenes* isolates had at least one virulence gene regardless of their source. Comparatively, all five virulence genes (*sdaB, speB, speJ, speA* and *ssa*) were commonly detected in *S. pyogenes* isolates collected from non-invasive samples but not the invasive samples, which could be due to a slightly higher number of non-invasive samples in the present study (Table I). Nonetheless, high-frequency rates of similar virulence genes have also been reported among *S. pyogenes* strains from non-invasive samples in previous studies.[23,24] This is not surprising as the distribution of virulence genes varies due to the differences in the acquisition of the genes which can be chromosomally encoded or mediated by mobile genetic elements.[25] Both *streptococcal pyrogenic exotoxin A (speA)* (65.0%) and *streptococcal superantigen (ssa)* (88.9%) genes are highly transmissible via horizontal gene transfer through mobile genetic elements.[25] The emergence of phage-encoded exotoxins would create an unusual virulent clone among certain population due to selective pressures for bacterial fitness.[24] Moreover, the lack of specific immunity among the study population towards such strains may pose a substantial risk of a streptococcal outbreak in future. Similar findings have been reported where high frequency of *speA* gene was detected in non-invasive cases (pharyngitis) in Taiwan and Norway.[26,27] However, lower frequency rates have been reported in recent studies in Pakistan (38%) and India (25.1%).[24,28] The SpeA, SpeJ and SSA toxins have been commonly associated with severe *S. pyogenes* infections such as toxic shock-like syndrome, multiorgan failures and scarlet fever.[29] SpeB, a cysteine protease is a potent pro-inflammatory inducer and is commonly associated with necrotising fasciitis.[29] In the present study, a slightly low frequency of *speB* gene (61.9%) was noted from the analysis of *S. pyogenes* isolates. The *speB* gene was shown to be highly conserved in *S. pyogenes* isolates (100%) in some studies[28,30] while few studies have reported lower frequency rates ranging from 0 to 60%.[25,31] The differences in the prevalence rates of *speB* gene could possibly be explained by the different strains in certain geographical regions, the number and types of samples and the methods used for *speB* gene detection. Interestingly, streptococcal DNase B *(sdaB)* gene was frequently detected among non-invasive than invasive samples in this study (54.5% versus 45.5%) and only absence in isolates collected from HKL. This finding can be associated with the common observation in phylogenetic tree. The *sdaB* gene has been designated as a *streptococcal pyrogenic exotoxin F (speF)* gene that could directly damage pulmonary endothelial cells in a mouse model.[32] This chromosomally encoded gene causes increased permeability of lung blood vessels which is a risk factor for acute respiration distress syndrome (ARDS) cases.[32] The high-frequency rate of *sdaB/speF* gene (100%) among invasive strains was reported in a few studies.[24,30] Inversely, lower frequency rates of *sdaB/speF* gene were reported in recent studies.[28,33]

A diverse distribution of *emm* types/subtypes among *S. pyogenes* was observed, in which 27 *emm* types/subtypes were detected (Table III). No new *emm* types/subtypes were detected. The results obtained align with other findings which reported no dominancy of a single *emm* type/subtype.[34,35] Moreover, it has been documented that *S. pyogenes* strains in developing countries have diverse *emm* types compared to developed countries.[36] The *emm* types/subtypes were also widely distributed among invasive and non-invasive samples in the present study which is inconsistent with findings from other studies where certain *emm* types (*emm*1, 3, 6, 12 and 89) were specifically found in invasive strains.[1,37] In general, the frequencies of five virulence genes were higher in non-invasive than invasive isolates (Table 11) and serious attention is needed as phage-encoded superantigens in non-invasive *S. pyogenes* strains can easily be transferred to invasive *S. pyogenes* strains and other non-pathogenic streptococci via horizontal gene transfer.[28] Nevertheless, the findings from this study are in contrast with other previous findings where a higher percentage of virulence genes was noted in invasive than non-invasive samples.[7,28]

In the present study, few prominent clusters with restricted patterns of virulotypes were observed by phylogenetic analysis (Figure 2). The findings are in accordance with a study that demonstrated certain *emm* types shared the common toxin gene profiles.[24] Balaji and colleagues reported that different *emm* types had distinct toxin-gene profiles but a phylogenetic analysis was not investigated among their isolates.[24] The spe genes provide as marker for horizontal gene movements and encode the function of exotoxin in GAS pathogenesis. Surveillance that includes invasive (tissue sources) and non-invasive (pus) GAS isolates is important to distinguish between virulence properties and the prevalence of a particular GAS strain in the general population and to evaluate epidemiological changes in GAS diseases.[24]

Specific dominant virulotypes (A and L) were prevalent in both hospitals, and close monitoring via molecular typing methods is urgently required. Toxin gene profiling (virulotyping) has been proposed in many studies to support other molecular typing methods such as the MLST for genotypic determination of *S. pyogenes*.[7,32] Besides, it was reported that several *emm* types have specific toxin gene profiles which were reflected by the spread of specific invasive clones in European countries.[7,32] The *emm*1/ST28 isolates that exhibited virulotype A and L in this study could pose a risk of clonal transmission in both hospitals. Hypervirulent characteristic of *emm*1/ST28 strain is typically associated with high fatality rates among invasive streptococcal cases in some countries.[38,39] The different *emm*/ST types that exhibited virulotype A in this study could be explained by the restricted or lack of transfer of phage-encoded superantigen genes within this clone. It is still unknown whether a single virulotype detected in a clone could be due to the underlying biological factors or the selective advantage of *S. pyogenes* strains with certain *emm*/ST types. Nonetheless, different patterns of toxin gene profiling within multiple *emm* types are usually reflected by ongoing horizontal transfer of phage-encoded superantigen genes over time.[40] Thus, continuous molecular surveillance is needed to identify the emergence of novel lineages of *S. pyogenes* local strains.

This study has several limitations. The total sample was too small in the present study for the statistical analysis to be carried out resulting in findings from this study could not be generalised to other Malaysian hospitals. However, as this study in the first report on virulence characteristics of *S. pyogenes* in Malaysia, it is very important to investigate the current

molecular epidemiology of the local strains for controlling the potential clonal spread of *S. pyogenes* isolates in hospitals across the country.

CONCLUSION

S. pyogenes is genetically diversified, and apart from the emm typing and MLST methods, virulotyping is essential to characterise the heterogeneous nature of *S. pyogenes* strains. Future study with a larger sample number of *S. pyogenes* isolates from different sources is needed to support the findings from this study. Continuous monitoring of *S. pyogenes* via molecular methods is warranted in the future. Thus, potential nosocomial outbreaks of invasive clones can be controlled accordingly.

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