Characterisation of the Virulence Factors and Genetic Types of Methicillin Susceptible *Staphylococcus aureus* from Patients and Healthy Individuals

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Received: 7 March 2012 / Accepted: 4 June 2012 / Published online: 20 June 2012
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Abstract Methicillin sensitive *Staphylococcus aureus* is an important bacterial pathogen associated with hospital- and community-acquired infections leading to endocarditis, skin tissue infection and pneumonia. The objective of this study was to determine both the genetic characteristics of methicillin-sensitive *S. aureus* (MSSA) strains, and the occurrence of virulence factors produced by *S. aureus* strains isolated from UMMC and healthy students in the University from year 2009. Out of 429 nasal swab samples, 67 were MSSA. The prevalence of 21 different virulence genes among 67 Malaysian clinical and community MSSA strains was determined by PCR, and their genetic features were assessed by PCR-RFLP of *coa* gene, *agr* types, *spa* typing and PFGE. The five predominant virulence genes were *ica* (79 %), *efb* and *fnbA* (61 % each), *sdrE* (57 %) and *hlg* (45 %). Toxin genes (enterotoxin, *etd* and *pvl*) were significantly more common ($P < 0.05$) in clinical strains compared to community strains. Three *agr* genotypes were observed: *agr* type I (45 %), *agr* type III (25 %) and *agr* type II (19 %). All 67 MSSA strains were distinguished into 26 profiles by PCR-RFLP of *coa*, 55 pulsotypes and 21 *spa* types. Four novel *spa* types (t7312, t7581, t7582 and t7583) were observed. In conclusion, different

virulence profiles were observed in MSSA strains in Malaysia where toxin genes were more prevalent among clinical strains. No correlation between DNA profiles (*coa*-RFLP, PFGE and *spa*) and virulotypes was observed. The Malaysian MSSA strains from clinical and community sources were genetically diverse and heterogeneous.

Keywords *Staphylococcus aureus* · Virulence genes · PFGE · *spa* and *agr*

Introduction

Staphylococcus aureus is one of the most important bacterial pathogens responsible for food-borne poisoning and toxin-mediated disease [1]. It produces different types of virulence factors, which are involved in attachment, persistence, tissue penetration and sepsis [2].

Enterotoxins are short secreted proteins that are usually heat-resistant, and usually resistant to most of the proteolytic enzymes produced by the human body [3]. There are more than 20 different types of enterotoxins (SEA to SEE, SEG to SEI, SEIJ, SEIK-SEIQ, SER to SET, SEW, SEIU) being reported [4]. An accessory gene regulator (*agr*) is known to be a global regulator of staphylococcal virulon which coordinates the expression of secretion and cell-associated virulence factors [5].

MRSA has evolved from methicillin-susceptible *S. aureus* (MSSA) via acquisition of mobile genetic elements called staphylococcal cassette chromosome *mec* (SCC*mec*) [6, 7]. Hence; MSSA is known to be a potential reservoir for these MRSA strains.

Several methods are available for subtyping *S. aureus*, include PCR-RFLP of *coa* gene [8], pulsed-field gel electrophoresis (PFGE) [9], and *spa* typing [10]. PCR-RFLP of

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coa gene is based on the restriction digest of the heterogeneity of the coagulase region that contains the 81 bp tandem repeats at 3' coding region [11] whereas PFGE is based on digestion of chromosomal DNA with the restriction enzyme followed by agarose gel electrophoresis [12]. On the other hand, *spa* typing is based on the sequence analysis of polymorphic region X of *S. aureus* protein A is reported to be a highly effective tool in subtyping *S. aureus* [12].

Detailed report on the genetic characterisation of MSSA strains in Malaysia is scanty. The only report in Malaysia was carried out by Ghasemzadeh-Moghaddam et al. [13] who reported diverse genotypes of MSSA strains isolated from six different populations of diverse geographical areas.

The objective of this study was to determine both the genetic characteristics of methicillin-sensitive 67 *S. aureus* (MSSA) strains, and the occurrence of virulence factors produced by MSSA strains isolated from UMMC and healthy students in the University.

Materials and Methods

Bacterial Strains

Sixty-seven non-repeat MSSA strains obtained from 29 in-patients admitted to University Malaya Medical Centre (UMMC) and 38 healthy undergraduate students from University Malaya (UM) in year 2009 were studied. The 38 MSSA strains from healthy students were collected from *S. aureus* carrier study involving 400 volunteer students.

All strains were isolated from nasal swabs. The strains were identified by standard biochemical methods, confirmed by BBL Staphyloside-Latex Test (BD, USA) and were checked for purity before analysis. All the strains were cultured in Tryptone Soy Broth and stored in cryo-vials containing 25 % v/v glycerol (Invitrogen, USA) at -85°C .

PCR Detection of Virulence Genes

Detection of 21 different virulence genes including enterotoxins, *sea* to *see*, *seg* to *sej* and *tst* [14]; exfoliative-toxin, *eta*, *etb* [14] and *etd* [15]; cytotoxin, *pvl* [16]; adhesion genes, *cna*, *hlg*, *ica* and *sdrE* [17], *efb* [18], *fnbA* and *fnbB* [19] were performed by PCR using conditions as described earlier [14–19].

agr Typing by Multiplex PCR

Multiplex PCR for *agr* types was performed using specific primers and conditions described by Lina et al. [20].

Genotyping of MSSA Strains by PCR-RFLP of *coa* Gene, PFGE and *spa* Typing

PCR amplification of *coa* gene was performed using genomic DNA, primers and cycling parameters as described by Hookey et al. [21]. The amplicon of *coa* was digested with 10U of *AluI* enzyme (Promega, USA) for 1-h at 37°C and the digested products were separated in 1.5 % agarose gel at 90 V for 3-h.

PFGE was performed according to Thong et al. [9]. The chromosomal DNA was digested with 10U *SmaI* for 2-h followed by separation on CHEF DRIII (Bio-Rad, USA) in $0.5\times$ Tris–borate–EDTA at 14°C for 22-h with pulsed-time of 5–60 s. Both PCR-RFLP of *coa* gene and PFGE gels were photographed under UV light after staining with ethidium-bromide (0.5 $\mu\text{g/ml}$).

Cluster analyses of PCR-RFLP for *coa* gene and PFGE profiles of MSSA strains were analyzed with BioNumerics 6.0 (Applied Maths, Belgium) based on unweighted-pair group method with arithmetic averages (UPGMA) with a position tolerance of 0.15. All DNA profiles were assigned arbitrary designation, and differences were defined by Dice coefficient of similarity, *F*.

spa typing was performed as described by Harmsen et al. [22]. The amplicons of *spa* were purified using Megaquick-DNA purification kit (Intron Biotechnology, Korea) and sequenced. Nucleotide sequences of *spa* type were analyzed using BioNumerics 6.0.

Cluster analysis setting for minimum-spanning tree was set to 25 % duplicate extension, 25 % duplicate creation, 50 % gap extension cost, 250 % gap creation cost, maximum duplication length of three-repeats and bin grouping distance of 0.5 %. Based on the interpretation scheme recommended by Shore et al. [23], two strains are considered closely related if two *spa* types are at a MST distance of ≤ 3 (corresponding to $>98.5\%$ similarity).

Statistical Analysis

Statistica 8.0 software was used for data analysis. Comparison of certain variables was determined by Fisher's exact test. Associations between different virulence factors were determined by Spearman's rank order correlation coefficient test. *P* value <0.05 (two-tailed) was taken as the level of significance for Fisher's exact test whereas *R*-value was taken as type of association between the variables. Breakpoints for the association of virulence factors are defined as follows: perfect association ($R = 1$), no association ($R = 0$) and invert correlation with ($R = -1$).

Table 1 Prevalence of virulence genes, *agr* and *spa* types in Malaysian MSSA strains

Gene	Clinical (n = 29)	Community (n = 38)	P value
Enterotoxin			
<i>sea</i>	6	3	0.1604
<i>seb</i>	3	0	0.0763
<i>sec</i>	6	5	0.5116
<i>seg</i>	3	0	0.0763
<i>seh</i>	9	3	0.0229
<i>sei</i>	6	3	0.1604
<i>etd</i>	0	1	1.0000
<i>tst</i>	1	5	0.2235
Adhesion			
<i>Efb</i>	18	23	1.000
<i>fnbA</i>	18	23	1.000
<i>hlg</i>	14	16	0.6300
<i>ica</i>	24	29	0.5613
<i>sdrE</i>	17	21	0.0806
Cytotoxin			
<i>pvl</i>	2	7	0.2802
<i>agr</i> types			
<i>agr</i> type I	9	21	0.0818
<i>agr</i> type II	4	9	0.3650
<i>agr</i> type III	11	6	0.0503
Un-typeable	5	2	0.2251

Results and Discussion

Prevalence of Virulence Genes Among MSSA Strains

Among the 21 virulence genes tested, *ica* gene was detected in a majority (79 %) of the strains. *efb*, *fnbA*, *sdrE* and *hlg* genes were detected in 41 (61 %), 41 (61 %), 38 (57 %) and 30 (45 %) strains, respectively. Based on Spearman's rank correlation test, correlations between *efb* and *fnbA* ($R = 1$, $P < 0.05$), *fnbA* and *sdrE* ($R = 0.478$, $P < 0.05$), *fnbA* and *hlg* ($R = 0.71$, $P < 0.05$), *fnbA* and *ica* ($R = 0.118$, $P < 0.05$), *fnbA* and enterotoxin ($R = 0.38$, $P < 0.05$) were observed. This indicates that *efb*-positive strains are most likely to harbour *fnbA*, *ica*, *hlg*, *sdrE* and enterotoxin's genes. Presence of *efb*, *fnb*, *ica* and *hlg* genes are needed for pathogenesis and adherence of *S. aureus* in host tissue [2].

A total of 39 strains was tested positive for at least one type of staphylococcal enterotoxins, *etd* or *pvl* gene. No *sed*, *see*, *sej*, *eta*, *etb* and *etd* gene was detected. The distribution of virulence genes in community and clinical MSSA strains is summarized in Table 1. There was a significant difference in the prevalence of *seh* gene between clinical and community strains ($P = 0.0229$), and

this concurred with the result reported by Ghasemzadeh-Moghaddam et al. [13]. There was no significant difference in the prevalence of other virulence genes, *efb*, *fnbA*, *hlg*, *ica*, *sdrE*, *sea*, *seb*, *sec*, *seg*, *sei*, *etd*, *tst* and *pvl*, between clinical and community strains. However, Ghasemzadeh-Moghaddam et al. [13] reported that *sea*, *seb*, *seg*, *sei* and *fnbA* were significantly more prevalent among community than clinical strains. The discrepancy between the distribution of these six virulence genes might be related to the sources of the community strains as the previous study examined strains from six different populations, including aboriginals, university student and staff, kindergarten kids and village people whereas the present study only examined community strains from healthy undergraduate students from one institution.

PVL toxin (encoded by *pvl* gene) that causes necrotic suppurative skin lesions [24] was common among community than clinical strains. This is not surprising as *pvl* gene is often associated with community strains (MRSA and MSSA) and is considered as a marker for community-acquired infections [6].

Toxic shock syndrome toxin-1 (encoded by *tst*) that causes neonatal toxic shock syndrome-like exanthematous disease and *Staphylococcal purpura fulminans* [25, 26] was detected in five community and one clinical strain.

The number of MSSA strains harbouring toxin genes were significantly higher ($P < 0.05$) in clinical strains when compared to community strains. This is a cause of concern as enterotoxins are often associated with food poisoning and several allergic and autoimmune diseases [1, 3].

Occurrence of *agr* Types

Three *agr* types were observed: *agr* type I (45 %), *agr* type II (25 %) and *agr* type III (19 %). No *agr* type IV was observed. Seven strains did not belong to any *agr* type. This concurred with the report by Perez-Vazquez et al. [27] that *agr* types in MSSA were more diverse as 35 (31 %), 40 (35.4 %), 35 (31 %) and 3 (2.7 %) of their MSSA strains were subtyped into *agr* types I, II, III and IV. Besides, no significant difference between the distribution of *agr* types in clinical and community MSSA strains were observed.

Genetic Relatedness of MSSA Strains based on PCR-RFLP of *coa* Gene, PFGE and *spa* Typing

Three different typing approaches were used to subtype the 67 MSSA strains. Based on PCR-RFLP of *coa*, the 59 strains were subtyped into 27 different restriction profiles ($F = 0.3$ – 1.0) (Fig. 1), eight strains could not be typed due to the absence of *coa* gene. Sixteen strains were genetically related (shared 80 % similarity) and among

Fig. 1 Dendrogram of PCR-RFLP of *coa* gene of clinical and community MSSA strains

Fig. 2 Dendrogram of PFGE *SmaI*-digested chromosomal DNA profiles of clinical and community MSSA strains

them, 12 strains were indistinguishable, although they were from two different sources (clinical and community) (Fig. 1).

PFGE differentiated the 67 strains to 55 pulsed-field profiles (PFs) ($F = 0.54\text{--}1.0$) (Fig. 2) with 11 strains (4 clinical, 7 community) being clonally related (shared $\geq 80\%$

Fig. 3 Minimal spanning tree analysis for the *spa* types of 67 MSSA strains

similarity). Different virulence profiles were observed for the clonally related strains, suggesting that presence of enterotoxin and cytotoxin genes were not associated with any particular PFGE profiles.

The 67 MSSA strains were subtyped into 21 different *spa* types, including four novel *spa* types (t7312, t7581, t7582 and t7583) (Fig. 3). Seven *spa* types (t084, t1272, t177, t127, t376, t359 and t037) were present in both clinical and community strains. Five other *spa* types (t7583, t337, t7582, t189 and t4720) were unique for clinical strains while nine *spa* types (t3169, t548, t131, t6912, t7312, t3380, t4236, t7581 and t050) were only present in community strains. *spa* types t037, t127, t131, t177, t050 and t189 have been reported elsewhere [13, 28] while *spa* types t3169, t376, t6912, t337, t4236, t3380, t7312, t7581, t7582 and t7583 are new in Malaysia (<http://ridom.spa.server.com>).

A minimum spanning tree based on the degree of similarity between *spa*-type repeats successions showed four *spa* clonal complexes (*spaCC*) arbitrarily named *spaCC*1 to *spaCC*4 (Fig. 3). Fourteen strains from four *spa* types (t7312, t189, t6912 and t3380) were closely

related (shared ≥ 98.5 % similarity), and all four *spa* types shared three *spa*-type repeats succession (12-21-17) although they only shared 60.2 % similarity by PFGE. Different virulence profiles were observed for strains with indistinguishable profiles, suggesting that presence of enterotoxin and cytotoxin genes were not associated with any particular *spa* types. This is because virulence genes such as enterotoxin genes are often carried by mobile genetic elements such as plasmids, pathogenicity islands, *SCCmec* and prophages [29].

Both PFGE and *spa* typing were more discriminative than PCR-RFLP of *coa* gene. Twelve strains that exhibited identical PCR-RFLP of *coa* profiles were distinguishable by *spa* typing and PFGE. Discrepancies in the grouping of the strains by the three subtyping methods might be due to different target sites as PCR-RFLP target the *coa* gene, *spa* typing examines the variability in *spa* gene whereas PFGE targets large genomic fragment after digestion with restriction enzymes.

In conclusion, different virulence profiles were observed in MSSA strains in Malaysia where toxin genes were more

prevalent among clinical strains. However, virulence genes studied were not source-specific and no correlation between DNA profiles (i.e. PCR-RFLP of *coa* gene, PFGE and *spa* types) and virulotypes was observed. The Malaysian MSSA strains from clinical and community sources were genetically diverse and heterogeneous.

Acknowledgments This work was funded by PPP Grant (PV046/2011B) from University of Malaya and PulseNet Grant (57-02-03-1015) from JJID, Japan. LKT is supported by University of Malaya Fellowship.

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