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Investigation of toxin genes among methicillin resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia

Lim, K.T.[1,2], Hanifah, Y.A.[3], Mohd Yusof, M.Y.[3] and Thong, K.L.[1,2*]
[1]Microbiology Division, Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur
[2]Biomedical Science and Molecular Typing Laboratory, A407, Institute of Graduate Studies, University of Malaya, 50603 Kuala Lumpur
[3]Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur
[*]Corresponding author email: thongkl@um.edu.my
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Abstract. *Staphylococcus aureus* is a persistent human pathogen responsible for a variety of infections ranging from soft-tissue infections to bacteremia. It produces a variety of virulence factors which are responsible for specific acute staphylococcal toxaemia syndromes. The objective of this study was to determine the prevalence of a repertoire of toxin genes among Malaysian MRSA strains and their genetic diversity by PCR-RFLP of *coa* gene. One hundred eighty-eight strains (2003, 2004, 2007 and 2008) of methicillin-resistant *S. aureus* (MRSA) were screened for 20 genes encoding for extracellular virulence determinant (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *tst*, *eta*, *etb*, *etd*) and adhesins (*cna*, *etb*, *fnbA*, *fnbB*, *hlg*, *ica*, *sdrE*). The genetic relatedness of these strains was determined by PCR-RFLP of *coa*gene and *agr* grouping. Majority of the strains were tested positive for *efb* and *fnbA* (96% each), *ica* (78%) and *hlg* (59%) genes. A total of 101 strains were positive for at least one type of staphylococcal enterotoxin genes with *sea* being the predominant. Genes for *seb*, *sed*, *see*, *seh*, *sej*, *eta* and *etb* were not detected in any of the MRSA strains. The prevalence of *sea*, *sec* and *ica* among strains isolated in 2008 was increased significantly ($p< 0.05$) compared to 2003. Most of the strains were of *agr* type I (97.5%) followed by *agr* type II (1.2%) and *agr* type III (0.6%). All *sea*, *sei* and *tst* gene-positive strains were of *agr* type I. The only *etd* positive strain was *agr* type III. PCR-RFLP of *coa* produced 47 different patterns. The number of strains with virulence factors (*sea*, *sec* and *ica*) had increased over the years. No direct correlation between PCR-RFLP-*coa* profiles and virulotypes was observed.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important bacterial pathogen responsible for suppurative and toxins-mediated disease (Ferry *et al.*, 2005). *Staphylococcus aureus* is known to produce a variety of virulence factors such as the staphylococcal enterotoxins (SEs), exfoliative toxins (Ets) and toxic shock syndrome toxin (TSST) which are responsible for specific acute staphylococcal toxaemia syndromes, including staphylococcal food poisoning and scalded skin syndrome (Mohamad Adwan *et al.*, 2006; Udo *et al.*, 2009).

Both SEs and TSST are members of the superantigenic toxin family that stimulate nonspecific T-cell proliferation (Ferry *et al.*, 2005; Ortega *et al.*, 2010; Demir *et al.*, 2011). A total of 18 different types of enterotoxins such as SEA-SED, SEE, SEG-SER and SEU encoded by *sea-sed*, *see*, *seg-ser* and *seu* genes, respectively have been reported (Ferry *et al.*, 2005). Among them, enterotoxin genes *seg*, *sei*, *sem*, *sen*, *seo* are located at enterotoxin gene cluster (*egc*) (Ferry *et al.*, 2005).

An accessory gene regulator (*agr*) is known to be a global regulator of the staphylococcal virulon which coordinates the expression of secreted and cell-associated virulence factors (Traber *et al*., 2008). There are four major *agr* types (designated *agr* type 1 to *agr* type 4) in *S. aureus* (Lina *et al*., 2003).

Molecular subtyping methods such as pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing are considered as 'gold standard' in typing MRSA strains, although these typing methods are often time-consuming, expensive and laborious (Ishino *et al*., 2006). An alternative simpler typing method, PCR-restriction fragment length polymorphism (RFLP) typing of the coagulase gene (*coa*) has been used in subtyping of MRSA strains as it is simple, rapid and inexpensive (Himabindu *et al*., 2009).

The objective of this study was to determine the prevalence of a repertoire of toxin genes among 188 Malaysian MRSA strains isolated in 2003, 2004, 2007 and 2008. The genetic relatedness of these strains was determined by PCR-RFLP of *coa*gene and *agr* grouping.

MATERIALS AND METHODS

Bacterial strains

A total of 188 non-repeat *S. aureus* strains from different patients admitted to University Malaya Medical Centre, Malaysia, in years 2003, 2004, 2007 and 2008 were studied. The organisms were isolated from respiratory samples [such as nasal swabs (n = 43; 22.9%), sputum (n = 23; 12.2 %) and nasopharyngeal secretion (n = 9; 4.8%)], tissue (n = 16; 8.5%), wound swabs (n = 34; 18.1%), urine (n = 6; 3.2%), pus (n = 12; 6.4%), body fluids (n = 24; 12.8%), catheter tip (n = 3; 1.6 %), bone (n = 4; 2.1%), blood (n = 13; 6.9%), chest tube "drainage" (n = 1; 0.5%).

All the strains were cultured in Luria-Bertani broth and stored in cryovials with 50% glycerol (Invitrogen, USA) at -20ºC and -85ºC.

PCR detection of virulence genes

Detection of 20 virulence genes (*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej*, *eta*, *etb*, *etd*, *tst*, *efb*, *fnbA*, *fnbB*, *cna*, *hlg*, *ica* and *sdr*) were performed by PCR using primers and cycling conditions as described earlier by Jarraud *et al.* (2002), Hisata *et al.* (2005), Arciola *et al.* (2005), Kumar *et al.* (2009), and Moore & Lindsay (2011). Genomic DNA from MRSA extracted by using Wizard Genomic DNA purification kit (Promega Madison Wis, USA) was used as DNA template.

Representative amplicons of *sea*, *sec*, *seg*, *seh*, *sei*, *etd*, *tst*, *efb*, *fnbA*, *ica*, *hlg* and *sdrE* were purified by using Qiagen DNA purification kit (Qiagen GmBH, Germany) and sequenced to validate their identities. All experiments were repeated once to confirm their reproducibility.

agr genotyping by multiplex PCR

Multiplex PCR for *agr* types was performed by using specific primers (*agr* type 1 to *agr* type 4) using conditions as described by Lina *et al.* (2003). Selected amplified products obtained were sequenced to validate their identity.

PCR-Restriction Fragment Length Polymorphism (RFLP) of *coa* gene

PCR amplification of *coa* gene was performed as previously described by Hookey *et al.* (1998) with minor modification. Briefly, PCR was carried out in a final volume of 25 µL containing 0.4 µM of each primer pair (Operon Biotechnologies GmbH, Germany), 35 µM of each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ and 0.5 U Taq DNA polymerase (Promega, Madison, Wis., USA).

The amplicon of *coa* was digested with *Alu*I enzyme (Promega Madison Wis, USA) as described by Hookey *et al.* (1998). Digested products were separated in 1.5% agarose gel at 90V for 3 hours. Gels were photographed under UV light after staining with ethidium bromide (0.5 µg/ml).

The banding patterns generated were analyzed using BioNumerics Version 6.0 (Applied Maths, Kortrijk, Belgium) and cluster analysis based on the unweighted pair group method with arithmetic averages (UPGMA) with a position tolerance of 0.15 was carried out. All DNA profiles were assigned arbitrary designation

and analyzed by defining a similarity (Dice) coefficient, *F*.

Statistical Analysis

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

RESULTS

Prevalence of virulence genes in MRSA strains

Majority of the strains were positive for the adhesion genes such as extracellular fibrinogen binding protein (*efb*)(96%), fibrinogen binding protein (*fnbA*) (96%), intracellular adhesion (*ica*) (78%) whilehemolysin (*hlg*) and putative adhesin (*sdrE*) were amplified in 59% and 27%, respectively. No collagen adhesin (*cna*) gene was detected.Based on Spearman's rank correlation coefficient test, correlation between *efb*and *fnb*(R=1, p < 0.05),*hlg* and *ica* (R=0.326, p < 0.05) were observed.

A total of 101 strains were tested positive for at least one type of staphylococcal enterotoxin (SEs) while one strain was positive for exfoliative toxin (*etd*). No enterotoxins (*seb*, *sed*, *see* and *seh*) or exfoliative toxins (*eta*, *etb*) gene was detected.Two strains harboured three SEs genes simultaneously whereas 30strains harboured two SEs genes (Table 1). The distribution of virulence genes is summarized in Table 1.

The occurrence of SEs and exfoliative genes had significantly increased between years 2003 and 2008 (p = 0.001). There was a significant increase in the prevalence of virulence genes, *sea* (p = 0.003), *sec* (p = 0.006) and *ica* (p = 0.010) in 2008 strains compared with 2003 strains. However, no significant difference in the prevalence ofvirulence genes in 2003 and 2008 strains for the following genes: *efb*, *fnbA*, *hlg*, *sdrE*, *seg*, *sei*, *etd* and *tst*.

sea and *sec* genes were detected in 17.5% and 6.9% MRSA strains and these strains were isolated from invasive samples (tissue,

Table 1. Prevalence of genes encoding virulence determinants in Malaysian MRSA strains in 2003, 2004, 2007 and 2008

Gene	No of strains		*p* value	Gene combination	No of strains	
	2003 to 2004	2007 to 2008			2003 to 2004	2007 to 2008
sea	19	54	*0.152*	*sea* + sec + sei		1
sec	4	32	*0.003*	*sec + seg + sei*		1
seg	1	11	*0.107*	*sec + sei*		7
sei	0	14	*0.006*	*sec + seg*		5
etd	0	1	*1.000*	*sea + sei*		2
tst	0	1	*1.000*	*sea + seg*	1	1
efb	59	121	*1.000*	*sea + sec*		14
fnbA	50	121	*1.000*	*sea*	18	36
hlg	34	75	*0.753*	*sec*	3	4
ica	39	104	*0.010*	*seg*		4
sdrE	14	37	*0.484*	*sei*		3
				etd		1
				tst		1
				Total	22 (36%)	80 (64%)

wound, blood, bone and pus) (Table 2). On the other hand, eight (67%) and ten (71%) MRSA strains isolated from colonization samples (sputum, nasal swabs and body fluids) were tested positive for *seg* and *sei* genes, respectively (Table 2).

Based on Spearman's rank correlation coefficient test, correlation between intercellular adhesion (*ica*) and SEs ($R=0.046$, $p < 0.05$), hemolysin (*hlg*) and SEs ($R=0.007$, $p < 0.05$) were observed.

agr genotyping

Three *agr* genotypes were observed: *agr* type I (97%; 51 strains from 2003, 9 from 2004, 15 from 2007 and 108 from 2008), *agr* type II (1.6%; 1 from 2007 and 2 from 2008) and *agr* type III (0.5%; 1 from year 2008). No *agr* type IV was observed. One strain (MRSA0312-35) did not belong to any *agr* group.

All three *agr* type II (MRSA0701-15, MRSA0806-14 and MRSA0812-36) strains harboured *seg* genes, and they were cultured from different sites and wards. The only *agr* type III strain (MRSA0806-13) which harboured *etd* gene was cultured from the nasal swab of a patient in the dialysis ward.

PCR-RFLP analysis of *coa* gene

Digestion of *coa* positive PCR products with *Alu*I enzyme yielded 47 different restriction profiles (F=0.24-1.0) (Figure 1). Four strains (MRSA0312-35, MRSA0704-15, MRSA0707-26 and MRSA0802-14) could not be typed by *coa*-RFLP typing despite, repeated attempts. Reproducible results were obtained in separate experiments using the same set of strains.

Seventy two MRSA strains were found to be clonally related as they shared more than 80% similarity. Among them, 37 strains shared identical PCR-RFLP profiles, even though they were cultured from different occasions and sources (Figure 1). Some strains from six years apart shared similar PCR-RFLP profiles. Further analysis showed that 31 out of these 72 MRSA strains (42.5%) harboured *sea* gene.

On the other hand, 33 strains were indistinguishable by *coa*-RFLP typing, although they were cultured from different sources and time periods (2003, 2004, 2007, 2008). Among them, 12 harboured *sea* genes, six had *sec* gene and one harboured *seg* gene.

DISCUSSION

Staphylococcus aureus is known to be responsible for a variety of toxins-mediated diseases (Ferry *et al.*, 2005). Although several studies have reported the incidence of selected virulence genes in MRSA in Malaysia (Ghaznavi-Rad *et al.*, 2010; Ghasemzadeh-Moghaddam *et al.*, 2011), reports comparing prevalence of virulence genes between two periods of times in Malaysia are scanty. This report shows detailed prevalence of virulence genes of MRSA isolated in a tertiary hospital in 2003, 2004, 2007 and 2008.

Table 2. Distribution of virulence genes in MRSA strains from different specimens

Gene	Number (%) of isolates (n=188)									
	Respiratory (n=75)	Tissue (n=16)	Wound (n=34)	Urine (n=6)	Pus (n=12)	Chest tube drainage (n=1)	Body fluid (n=24)	catheter tip (n=3)	Bone (n=4)	Blood (n=13)
sea	34	6	14	3	3		2	1	2	8
sec	21	4	5	1	2			1		2
seg	8	1	1						1	1
sei	12		1		1					
etd			1							
tst	1									
efb	70	16	33	6	11		24	3	4	13
hlg	36	13	19	2	8	0	21	1	4	6
ica	52	14	27	4	10	0	22	3	3	11
sdrE	30	2	7	2	6		1			3

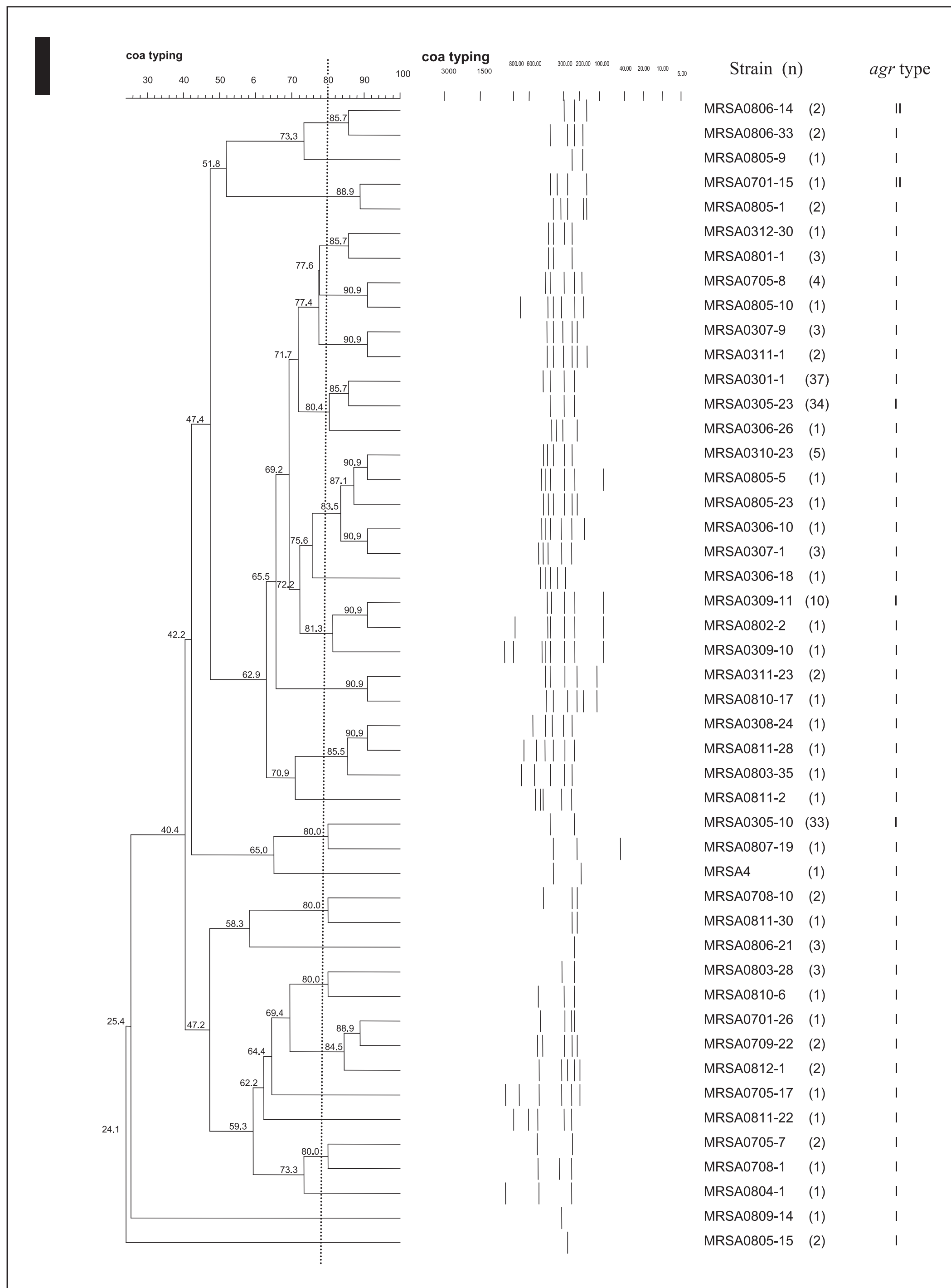

Figure 1. Dendrogram of PCR-RFLP of *coa* gene of MRSA strains. The dotted vertical line indicates 80% similarity level

Spearman's rank correlation analysis showed that there was a positive correlation between strains harbouring *efb* and *fnb* genes. Similarly, correlation between *hlg* and *ica* gene was observed. This is important as both *efb* and *fnb* genes are involved in the adherence of *S. aureus* strains while *ica* and *hlg* genes are involved in biofilm formation

and promotes host cell lysis, respectively (Ferry *et al*., 2005).

Overall, 54% of the MRSA strains harboured at least one type of SEs gene with the percentage of 2007 and 2008 strains harbouring SEs (62%) higher than 2003 and 2004 strains (36%). This increase might be caused by horizontal gene transfer among the strains as SEs genes are carried by mobile genetic elements such as plasmids, pathogenicity islands, *SCCmec* and prophages (Hu *et al*., 2008). This is of public health concern as SEs genes are often associated with food borne poisoning, toxic shock syndrome and other toxin mediated disease (Ferry *et al*., 2005; Ortega *et al*., 2010).

sea gene was the most common SEs gene present among Malaysian MRSA strains and this concurred with the finding reported from another tertiary hospital in Kuala Lumpur (Ghaznavi-Rad *et al*., 2010). However, this differed from a report by Sauer *et al.* (2008) where *seg* and *sej* genes were predominant in the MRSA strains in a University Hospital, Czech Republic. Although both *seg* and *sei* genes are located in the same *egc* operon (Sauer *et al*., 2008), most of the strains (99%) did not harbour these genes simultaneously ($R= -0.923$, $p < 0.05$) and this concurred the result reported by Collery *et al.* (2008). Only one invasive strain (MRSA0805-10) harboured *sec*, *seg* and *sei* genes simultaneously.

Exfoliative toxin (encoded by *etd*) that can cause an inflammatory response of the skin was detected in a MRSA0806-13 strain which belong to the *agr* type III. No *eta*, *etb* or *seb* genes were present in the UMMC strains and other Malaysian MRSA strains (Ghaznavi-Rad *et al*., 2010).

Although Sabat *et al*. (2006) reported that the absence of *sdrE* and *sdrD* genes in *S. aureus* might decrease its invasive potential in bone tissues, our result showed that indigenous *S. aureus* in this tertiary hospital can cause bone infections even without the presence of *sdrE* gene. This infection could have involved other alleles of the *sdr* gene.

Fifty (27%) invasive strainswere found to harbour hemolysin gene (*hlg*). This is not surprising as the previous report by Peacock *et al.* (2002) indicated that virulence factors such as *fnbA*, *cna*, *sdrE*, *hlg*, *sej*, *eta* and *ica* were significantly more common in invasive strains, and they contributed independently to virulence.

Significant increase in the occurrence of *ica*, *sec* and *sei* genes among 2008 strains when compared to 2003 strains was observed. Furthermore, Spearman's rank correlation tests also showed that MRSA strains with *ica* and *hlg* genes showed higher virulence potential as these strains also harboured SEs, exfoliative toxin and *tst* genes. This is a cause for concern as the biofilm-associated bacteria is normally resistant to host immune systems and antimicrobial, and the presence of SEs will further weaken the host immune systems (Plata *et al*., 2009).

Majority (97%) of the strains were of *agr* type I and this is consistent with previous report by Peerayeh *et al.* (2009). Although Collery *et al.* (2008) reported that strains possessing *tst* gene are often associated with *agr* type III, our only *tst* positive strain (MRSA0802-19) was associated with *agr* type I.

Genotyping by PCR-RFLP of *coa* gene using *Alu*I enzyme showed that most of the MRSA strains were clonally related although they were cultured from different time periods. Our previous study (Lim *et al*., in press) on UMMC strains also indicated that most of the MRSA strains were clonally related by PFGE with five different MLST types (ST239, ST772, ST22, ST6 and ST1178). This suggests that some MRSA clones were found to be predominant in this tertiary hospital. Some other strains obtained from years 2003, 2004 and 2007 also shared similar patterns with 2008 strains, indicating the persistence of particular PCR-RFLP types in UMMC hospital. These strains were cultured from different patients' wards, and harboured different type of virulence genes (i.e different type of SEs) showing that MRSA strains in this study are able to acquire or lose SEs genes as these genes are carried by mobile genetic elements such as plasmids, pathogenicity islands, *SCCmec* and prophages (Hu *et al*., 2008).

In general, no direct correlation between PCR-RFLP profiles and virulotypes was

observed. Strains with identical PCR-RFLP profiles frequently belonged to different virulence patterns. Increase of MRSA strains with virulence factors over the years signal the potential loss of the usage of antimicrobial agents in treating MRSA infections as MRSA strains with virulence factors is normally resistant to host immune systems and other antimicrobial agents. The MRSA clinical strains from UMMC were mostly genetically related, suggesting that few predominant clones of the species are involved in infection. The data from this current study may act as reference for monitoring the prevalence of virulence among Malaysian MRSA strains over a longer period of times.

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