Temporal changes in the genotypes of methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary Malaysian hospital based on MLST, *spa*, and *mec*-associated *dru* typing☆

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**A B S T R A C T**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main bacterial pathogens responsible for nosocomial infections leading to pneumonia, bloodstream, skin, and soft tissue infections. The objective of this study was to investigate the genomic changes of MRSA in a tertiary hospital between the years 2003, 2004, 2007, and 2008. One hundred fifty-four MRSA strains were characterized by multilocus sequence typing (MLST), *spa*, and *mec*-associated *dru* typing. Among the 154 strains, 29 different *dru*, 15 *spa*, and 8 MLST types were identified. Seven sequence types (STs) (ST239, ST22, ST5, ST6, ST80, ST573, and ST241) were identified among 2007-08 strains, although only 2 STs (ST239 and ST20) were observed among 2003 strains. Clones ST239-0037-dt13g, ST22-1032-(dt10a and dt10aw), and 28 other MRSA clones being introduced in 2007-2008 have replaced the ST239-0037-dt13d, 14h, 13i, 13l, 15m, 15l, and 11al clones present in 2003. The predominant MLST clone, ST239 (90.3%), was further distinguished into 7 different *spa* types and 26 different *dru* types, including 17 novel *dru* types. Maximum parsimony tree based on *dru* repeats revealed that 10 *dru* types (dt11am, dt13j, dt15n, dt13q, dt13n, dt13p, dt13f, dt13ao, dt12j, dt7v) shared the same MLST-*spa* types with dt13d, suggesting that these MRSA clones might have evolved from ST239-0037-dt13d. In conclusion, our data showed that the ST239-0037-dt13d clone and other MRSA clones in 2003 were replaced by ST239-0037-dt13g and other new emerging *spa* and *dru* types.

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1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the main bacterial pathogens responsible for nosocomial infections leading to pneumonia, skin and soft tissue infections, bloodstream infections, osteomyelitis, and endocarditis (Pérez Vázquez et al., 2009). MRSA evolved from methicillin-susceptible *S. aureus* via acquisition of mobile genetic elements called staphylococcal cassette chromosome mec (SCCmec) which has 2 essential components, the *ccr* gene complex and the *mec* gene complex (Chongtrakool et al., 2006).

Rapid and discriminative subtyping methods are essential to determine the epidemiology of pathogenic strains and are useful in the design of rational pathogen control methods. These methods include pulsed-field gel electrophoresis (PFGE) (Deurenburg et al., 2007), multilocus sequence typing (MLST) (Deurenburg et al., 2007; Enright et al., 2000), direct repeat unit (*dru*) typing (Goering et al., 2008) and *spa* typing (Deurenburg et al., 2007; Harmansen et al., 2003). MLST has been shown to be useful in global epidemiologic studies of *S. aureus* (Melles et al., 2007). *spa* typing, which is based on the sequence analysis of the polymorphic region X of the *S. aureus* protein A (*spa*) gene, is also a highly effective tool in subtyping *S. aureus* (Ruppitsch et al., 2006), whereas *mec*-associated *dru* typing could differentiate MRSA strains which were indistinguishable by PFGE analysis, including EMRSA15 and EMRSA16 (Goering et al., 2008; Shore et al., 2010). The advantages of both *spa* and *mec*-associated *dru* typing over MLST are their simplicity and relatively low costs as both typing methods only involve sequencing of a single locus as compared to MLST (Furuya et al., 2010; Shore et al., 2010). Furthermore, *mec*-associated *dru* typing can be used as a tool for phylogeny study of
MRSA as resistance in MRSA and other β-lactam antibiotics is mainly due to mecA gene situated on a mobile genetic element (SCCmec) (Deurenberg and Stobberingh, 2008).

The prevalence of MRSA in Malaysian hospitals has increased from 17% in 1999 to 26% in 2008 (Ministry of Health, 2008). The National Surveillance of Antibiotic Resistance Report in 2008 showed that the MRSA rates in 13 government hospitals ranged from 5.6% to 33.7% (Ministry of Health, 2008).

We have previously reported the emergence of ST22 (CC22) and the persistence of ST239 (CC8) in MRSA in the Thematic Medical Centre (UMMC), Malaysia (Lim et al., 2012). Ghaznavi-Rad et al. (2010) also reported that over 90% of MRSA strains isolated between 2007 and 2008 in another tertiary hospital (HKL) in Malaysia belonged to the pandemic clone ST239. Other MRSA clones reported in Malaysia were ST1 (CC1), ST7 (CC7), ST22 (CC22), ST188 (CC1), ST1283 (CC8), ST30 (CC30), ST45 (CC8), ST80 (CC80), ST101 (CC101), ST1285 (CC8), ST1286, ST1287, and ST1288 (Ahmad et al., 2009; Ghaznavi-Rad et al., 2010).

The predominant hospital-acquired MRSA (HA-MRSA) clones in 8 different Asian countries, including Taiwan, Korea, Hong Kong, Philippines, Vietnam, Sri Lanka, India, and Thailand, from September 2004 to August 2006 were ST5 and ST239 (Song et al., 2011). Similarly, this MLST ST239 was also found to be predominant in Russia and China (Baranovich et al., 2010; Xu et al., 2009).

Although several studies have documented the evolutionary changes of MRSA in other countries such as China, Germany, Portugal, and Iceland (Aires-de-Sousa et al., 2008; Chen et al., 2010; Holzknecht et al., 2008; Ghaznavi-Rad et al., 2010), there is no report, however, on the dynamics of MRSA over a long period in Malaysia. In addition, the understanding of molecular epidemiology of MRSA strains is important for any future efforts of controlling the spread and emergence of MRSA clones (Gadepalli et al., 2009). The objective of this study was to investigate the genomic changes of MRSA in a tertiary hospital in Malaysia between the years 2003, 2004, 2007, and 2008 based on MLST, spa, and mec-associated dru typing.

2. Materials and methods

2.1. Bacterial strains

Initially, all the MRSA strains from the 2003 to 2008 stock cultures were included in this retrospective study conducted in 2009. However, no MRSA strains from 2005 and 2006 were available as they were not viable. A total 154 non-repeat MRSA strains obtained from 150 patients and 4 healthcare workers were analyzed (43 from 2003, 9 from 2004, 16 from 2007, and 86 from 2008) from UMMC.

UMMC is a 980-bed referral and premier teaching hospital located in Klang Valley, Selangor, in Malaysia, which has surgical, orthopaedic, paediatric, and neurology ICUs. The organisms were isolated from nasal swabs (n = 34, 22%), tissue (n = 13, 8.4%), wound swabs (n = 30, 19.4%), urine (n = 6, 3.9%), pus (n = 8, 5.2%), body fluids (n = 19, 12.3%), sputum (n = 18, 11.7%), nasopharyngeal secretion (n = 7, 4.5%), catheter tip (n = 3, 1.9%), bone (n = 3, 1.9%), blood (n = 12, 7.8%), and chest tube “drainage” (n = 1, 0.6%). The strains were identified as MRSA by standard methods in the diagnostic microbiology laboratory of UMMC. Briefly, all the clinical specimens were streaked on blood and MacConkey agar and inoculated overnight at 35 °C. The suspected S. aureus colonies in blood agar (showed β-haemolysin with golden-yellow colonies) were further tested with the coagulase test and cefoxitin disk diffusion test following CLSI (2010) guidelines. The strains were identified as MRSA if zone diameter was ≤21 mm and tested positive in the coagulase test. The purity of the strains was checked with streaking on mannitol-salt agar before analysis. All the strains were cultured in tryptone-soya broth and stored in cryovials with 50% glycerol (Invitrogen, USA) at −20 °C and −85 °C.

2.2. spa and mec-associated dru typing

spa and mec-associated dru typing were performed on all 154 MRSA strains as described by Harmsen et al. (2003) and Goering et al. (2008), respectively. The amplicons of spa and dru were purified by using a commercial DNA purification kit (Bioneer, Korea) and sequenced to validate their identities. Nucleotide sequences of spa and dru amplicons and cluster analysis were analyzed using BioNumerics 6.0 software (Applied Maths, Belgium). The cluster analysis settings for the minimum spanning tree (MST) were set to 25% duplicate extension, 25% duplicate creation, 50% gap extension cost, 250% gap creation cost, maximum duplication length of 3 repeats, and bin grouping distance of 0.5%. Based on the interpretation scheme recommended by Shore et al. (2010), 2 strains are considered closely related if 2 spa or dru types are at a MST distance value of ≤3 (corresponding to >98.5% similarity). The distance between each node represents the similarity level between 2 entries, i.e., 2 entries that had a similarity of between 99.5% and 100% had a distance of 0.

The discriminatory power of spa, dru, and MLST typing was calculated based on Simpson’s index using the discriminatory power calculator available online (http://insilico.ehu.es/minitools/discriminatory_power/index.php).

2.3. Heteroduplex PCR for identification of the clone ST239 and MLST

Polymerase chain reaction (PCR) identification of the ST239 clone was performed on 154 MRSA strains as previously described by Feil et al. (2008), while MLST was conducted on all non-ST239 and 2 representative strains of ST239 as described by Enright et al. (2000). The allelic number and sequence types (STs) were assigned using the S. aureus MLST database (http://saureus.mlst.net), whereas clustering of related STs (defined as clonal complexes [CCs]) was analyzed with the BURST algorithm (http://eburst.mlst.net).

3. Results

3.1. Genotypes of MRSA based on spa types

Fifteen spa types were detected among the 154 strains. The most frequent spa type was t037 (72%). Repeats among other spa types varied from 1 strain (t002, t363, t458, t860, t1544, t4184, t1378, t2029, t4150, and t4152), 3 strains (t304), 4 strains (t4605), 5 strains (t032), and, finally, to 21 strains (t421) (Table 1). The discriminatory power of spa typing was 0.46.

Three spa types (t037, t421, and t1544) were present in 2003. Five spa types (t304, t4184, t002, t860, and t032) were detected in 2007, while 7 spa types (t1378, t4605, t4150, t4158, t4152, t2029, and t363) emerged in 2008 (Table 1).

A MST shows 2 spa clonal complexes (spaCC) arbitrarily named spaCC1 and spaCC2 (Fig. 1). Strains which shared more than 98.5% similarity were grouped in the same spa clonal complex (spaCC). spaCC1 consisted of 138 strains from 5 spa types (t037, t6405, t421, t363, and t2029) (Fig. 1). Among them, 58% (80/138) were from the year 2008. spaCC2 consisted of 7 strains from 3 spa types (t4184, t1378, and t032). The other 7 spa types (t1860, t4150, t1544, t458, t002, t304, and t4152) were not grouped in any spaCC as they shared less than 87.2% similarity.
Table 1


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3.2. Genotypes of MRSA based on mec-associated dru types

Sequence analysis of mec-associated dru in 154 strains gave 29 distinct dru types (Table 1). Eighteen novel dru types (dt13l, dt13m, dt11al, dt15i, dt7v, dt13n, dt15n, dt10aw, dt13ao, dt11am, dt13p, dt11an, dt13q, dt10ax, dt12j, dt12k, and dt14h) were detected. Information of these novel sequences was deposited at http://www.dru-typing.org.

The predominant dru type was dt13d (n = 48, 31.2%). Repeats among other dru types varied from 1 strain (dt12k, dt12j, dt11an, dt2c, dt13p, dt7l, dt10aw, dt15n, dt13i, dt15i, dt15m, and dt14h), 2 strains (dt13l, dt13m, dt7v, dt10ax, and dt11am), 3 strains (dt11c, dt13ao, and dt13q), 4 strains (dt11al, dt13f, 5 strains (dt9w, dt14c, dt13n, and dt13j), 16 strains (dt10a), and 31 strains (dt13g) (Table 1). The discriminatory power of mec-associated dru typing was 0.85.

Furthermore, the higher discriminatory power of mec-associated dru typing was able to differentiate spa type t037 into 23 different dru types (dt15m, dt15n, dt13p, dt12j, dt15l, dt13i, dt14h, dt11am, dt7v, dt13m, dt13l, dt13q, dt13ao, dt11c, dt13f, dt9w, dt13j, dt11al, dt13n, dt14c, dt10a, dt13g, and dt13d) (Table 1).

A MST based on dru repeats showed 4 dru clonal complexes (druCC) arbitrarily named as druCC1 to druCC4. druCC1 consisted of 100 MRSA strains with dru types dt13d, dt13j, dt13g, dt13n, dt13ao, dt13p, dt13m, dt13f, and dt13i, whereas druCC2 consisted of 3 strains with dru types dt15n, dt15l, and dt15m. Each of the smaller clusters, druCC3 and druCC4, consisted of 2 dru types (figure not shown).

3.3. Genotypes of MRSA based on MLST of 7 housekeeping genes

Of the 154 MRSA strains, 139 (42 from 2003, all 2004 strains, 8 from 2007, and 80 from 2008) produced both DNA bands (220 and 480 bp), as determined via the heteroduplex PCR. This indicates the presence of ST239 lineage (CC8). This was further confirmed by MLST.

Besides ST239, 7 different MLSTs including ST22 (CC22) (n = 7), ST6 (CC6) (n = 3), ST20 (CC20), ST5 (CC5), ST573 (CC15), ST80 (CC80), and ST241 (CC8) (1 each) were observed (Table 1). The discriminatory power of MLST was 0.18.

ST5, ST6, and ST22 were detected among MRSA strains from 2007. ST6 and ST22 were also present in MRSA strains from 2008. The other MLST types (ST573, ST80, and ST241) were only present in 2008. The predominant MLST clone, ST239, was further differentiated into 7 spa types (t037, t421, t6405, t4150, t4152, t2029, and t860) and 26 different dru types, including 17 novel dru types (dt14h, dt13i, dt13m, dt11al, dt15i, dt15m, dt7v, dt12j, dt13ao, dt13n, dt13p, dt13q, dt10ax, dt11am, dt12k, dt11an, and dt15n), whereas ST22 was differentiated by spa and mec-associated dru typing into 3 spa types (t032, t4184, and t1378) and 2 dru types (dt10a and dt10aw), respectively (Table 1).

3.4. Evolutionary changes in MRSA based on mec-associated dru typing

Thirteen dru types (i.e., dt9w, dt13i, dt13m, dt13g, dt13d, dt15m, dt15i, dt14h, dt14c, dt11al, dt10a, and dt11c) were detected in 2003. Two dru types (dt13n and dt7v) were introduced in UMMC in 2004. 3 dru types (dt10aw, dt15n, and dt13ao) in 2007, and 11 dru types (dt11am, dt12k, dt13q, dt13j, dt13p, dt13f, dt11an, dt10ax, dt12j, dt7l, and dt2c) were found only in strains from 2008 (Fig. 2; Table 1).

dt13d was found to be closely related to 8 other dt types (i.e., dt13f, dt13ao, dt13m, dt13g, dt13j, and dt13p) with differences in only 1 mutation, and all 9 dru types shared the same MLST type ST239, even though they were obtained from 4 years apart.

**Table 1** (continued)

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NPS = Nasopharyngeal secretion; WS = wound swab; NS = nasal swab; BD = body fluids; CT = chest tube; CCU = cardiac care unit.

**Fig. 1.** Minimal spanning tree analysis for the spa types of 154 MRSA strains. The clustering was performed by using the BioNumerics Spa Typing Plugin with a conservative alignment setting of 100% and 0% maximum duplication length. The numerals on the line indicate the similarity level between each spa type.

The combined analysis by MLST–spa types showed the genotypic changes of MRSA clones ST239-t037 and ST239-t421 between the years 2003 and 2008 where i) the predominant ST239-t037 clone in 2003 (95%) decreased in 2008 (66.3%), while ii) the ST239-t421 clone (2.3% in 2003) increased in 2008 (18.6%). It was observed that the ST239-t421 clone (from 2003) from the ICU might have spread to other wards, i.e., medical, dialysis, surgical, and orthopaedic in 2008 (Table 1). Both ST239-t037 and ST239-t421 clones cover 97.3% of strains from 2003 and 85% of strains from 2008.

Four new MLST–spa clones (ST22-t032, ST5-t002, ST6-t304, and ST22-t4184) were introduced in 2007 and another 3 MLST–spa clones (ST241-t363, ST80-t037, ST573-t1378) in 2008. All 4 MLST–spa clones that were introduced in 2007 were cultured from the medical, paediatric, and surgical wards, whereas newly introduced clones in 2008 were cultured from the orthopaedic and dialysis wards (Table 1).

Further combined analysis by MLST–spa–dru can increase the discriminatory power in the subtyping of MRSA strains. Forty-seven different MRSA clones were present in UMCC over the study period (2003, 2004, 2007, and 2008) where i) the ST239-t037-dt13d clone in year 2003 (48.8%) decreased in 2008 (14.0%); ii) the ST239-t037-dt14h, ST239-t037-dt13i, ST239-t037-dt13l, ST239-t037-dt13m, ST239-t037-dt13n, ST239-t037-dt15l, ST239-t037-dt15m, ST20-t1544-dt14c clones were present only in 2003 (20.9%); iii) ST239-t421-dt13d introduced in 2003 slightly increased in 2008 (4.7%); iv) the ST239-t037-dt13g clone in 2003 (4.7%) that was cultured from the orthopaedic and ICU wards spread to 6 other wards including the orthopaedic, ICU, surgical, medical, cardiac care unit, and paediatric wards, and became more dominant in 2008 (31.4%) (Table 1); v) ST22-t032-dt10a, ST6-t304-dt10a, and ST239-t037-dt13ao clones introduced in 2007 existed at low prevalence in 2008; vi) ST239-t037-dt13n and ST239-t037-dt17v clones were introduced in 2004; vii) ST5-t002-dt10a, ST22-t4184-dt10a, ST239-t037-dt13d, ST239-t037-dt15m, ST22-t032-dt10aw, and ST229-t421-dt13ao clones were introduced in 2007; and viii) 21 new clones were introduced in 2008 and they were cultured from 5 different wards including the dialysis, medical, orthopaedic, surgical, and ICU wards (Fig. 3; Table 1).

4. Discussion

Although several studies have documented the evolutionary changes of MRSA in other countries such as China, Germany, Portugal, and Iceland (Aires-de-Sousa et al., 2008; Chen et al., 2010; Holzknecht et al., 2010; Wisplinghoff et al., 2005), there is no report, however, on the longitudinal study of the genetic characteristics of MRSA in Malaysia. In order to understand the MRSA trends and their molecular evolution in a tertiary hospital in Malaysia, we characterized MRSA strains recovered in 2003, 2004, 2007, and 2008 by MLST–spa–dru typing methods.

Cluster analysis based on spa types showed that most (89.6%) of the MRSA strains were closely related, grouped in spaCC1 (Fig. 1), and they shared the same spa-repeat succession (02-25-17), while cluster analysis based on dru types indicated that over 64.9% of the MRSA strains studied were closely related with 2 dru types, dt13d and dt13g, being the predominant clones. Over 71.4% of the strains studied were of spa type t037, which implies the persistence of these spa types within the hospital environment. This suggests that some MRSA clones were circulating in this tertiary hospital during the study period.

Ghaznavi-Rad et al. (2011) has reported the occurrence of 6 dru types (dt10a, dt14c, dt13d, dt13i, dt9w, and dt13g) among strains from a tertiary hospital (HKL) in Malaysia during 2007–2008. However, our study indicates that these 6 dru types might have been disseminated in Malaysia earlier than expected as these 6 dru types had been detected in our strains since 2003.

The subtyping by MLST also showed that majority (140/154, 90.9%) of MRSA strains used in this study was associated with ST239. This ST239 clone is a single-locus variant of ST8, within clonal complex 8, which contains HA-MRSA clone ST241. Both clones differed by a single-point mutation in the yqil locus. Although MLST ST241 is novel in Malaysia, this genotype has been reported earlier in Germany (Deurenberg et al., 2005) and India (Gadepalli et al., 2009).
Ye also related to another MRSA clone (ST188) reported in 2007 with a single-point mutation in the characterized as SCC which is also known as Rhinne Hesse epidemic strain, was by Aires-de-Sousa et al. (2008) in Portugal. However, this MRSA ST5, shown). The presence of ST5 with SCC be associated with In this study, ST241, which was introduced in 2008, was also found to 2007, and 2008 in the UMMC, Kuala Lumpur, Malaysia.

The limitations of this study are as follows: the number of MRSA strains in the different study periods (2003, 2004, 2007, and 2008) were present throughout the study periods. Further combined analysis with MLST—spa—dru typing methods could further differentiate the ST239 clone into 7 spa types (t037, t421, t6405, t4150, t4152, t2029, and t860) and 26 different dru types. In another study, Ghaznavi-Rad et al. (2011) also showed that mec-associated dru typing is useful to enhance the epidemiologic discrimination of ST239. This suggests that the combination of spa and mec-associated dru typing together might be useful in subtyping MRSA as both spa and mec-associated dru typing are more rapid, less laborious, and relatively cheaper than MLST.

Our data demonstrated that the predominant ST239-t037 clone decreased from 95% in 2003 to 66.3%, while there was a slight increase in the prevalence of ST239-t421 from 2.3% in 2003 to 18.6% in 2008, suggesting the changes of MRSA clones in UMMC during the study period. Further combined analysis with MLST—spa—dru typing demonstrated that the prevalence rate of MLST239-t037-dt13d decreased from 48.8% in 2003 to 14.0% in 2008, and was replaced by MLST239-t037-dt13g and other new emerging MRSA clones that accounted for 70.9% of MRSA strains in 2008. ST239-t037-dt13g could easily be transmitted in this hospital as they have spread from 2 wards (in 2003) to 6 different wards (in 2008). Strains with dt13a types (dt13ao, dt13f, dt13p, dt13j, and dt13n) evolving from dt13d by 1 mutation, suggesting that some strains from these dt13a types might have originated from dt13d. Nevertheless, the mec-associated dru typing method cannot be applied on methicillin-sensitive S. aureus strains as they lack the mecA gene. In addition, mec-associated dru typing was unable to differentiate different lineages as dt10a consists of MRSA strains from 5 different lineages (CC5, CC6, CC22, CC8, and CC80). Therefore, mec-associated dru typing should be used in combination with another sequence typing method (i.e., spa typing) to characterize MRSA strains.

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Fig. 3. Distribution of different MLST—spa—dru types of MRSA strains from 2003, 2004, 2007, and 2008 in the UMMC, Kuala Lumpur, Malaysia.
being introduced in later years, suggesting that they might have evolved from dt13d and that dt15n might have possibly evolved also from dt15l. The combined analysis by MLST–spa–dru types indicated the changes of MRSA clones at UMMC in Malaysia where ST239–t037–dt13d and other MRSA clones in 2003 were replaced by ST239–t037–dt13t and other new emerging spa and dru types. Both spa and mec–dru typing are relatively cheaper, rapid, and have a greater discriminatory ability than MLST in subtyping of MRSA isolates. The data in this study underlined the necessity of surveillance typing in order to control MRSA strains in this hospital.

References