Original Article

Predominance of ST22-MRSA-IV Clone and Emergence of Clones for Methicillin-Resistant Staphylococcus aureus Clinical Isolates Collected from a Tertiary Teaching Hospital Over a Two-Year Period

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SUMMARY: Methicillin-resistant Staphylococcus aureus (MRSA) is one of the most common nosocomial pathogens, causing mild to severe infections. This study aimed to determine the genotypic and phenotypic characteristics of clinical MRSA isolates collected from a teaching hospital from 2014-2015. These isolates were genotyped by multilocus sequence typing, staphylococcal cassette chromosomal mec (SCCmec) typing, virulence genes detection, and pulsed-field gel electrophoresis; they were phenotyped based on their antibiotics susceptibility profiles. The most prevalent sequence type was ST22. ST3547 was identified from a blood isolate from 2015. Three SCCmec types (II in 26.26%, IV in 70.71%, and V in 3.03% isolates) were detected. Agr type I, II, and III were also detected among the isolates. The most prevalent virulence genes found were hemolysin (100%) and intracellular adhesion (91.9%). At least one staphylococcal enterotoxin was detected in 83 (83.8%) isolates. All the isolates were susceptible to vancomycin (minimal inhibitory concentration ≤ 2 μg/mL). Statistical analysis revealed a significant increase in hypertension (p = 0.035), dyslipidemia and obesity (p = 0.046), and previous exposure to any quinolone (p = 0.010) cases over the two-year period. The emergence and circulation of community-associated MRSA variants were observed in our hospital.

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) is an established nosocomial pathogen that causes infections, such as bacteremia, respiratory tract infections, skin and soft tissue infections (SSTIs), bone and joint infections, and urinary tract infections (1, 2). The prevalence rate of hospital-acquired MRSA infections was the highest (> 50%) in Asia, South America, and Malta, followed by lower prevalence rates (25 - 50%) in Australia, China, Africa, Romania, Greece, Italy, and Portugal (3). The prevalence rate of MRSA in Malaysia has been reported to increase from 17% in 1986 (4) to 44% in 2007 (5). To date, healthcare-associated MRSA (HA-MRSA) remains a global healthcare concern; however, the emergence of community-associated MRSA (CA-MRSA) since the 1990s poses significant risks and threats to both hospital and community populations. The current modality of treatment for MRSA infections is prescribing antibiotics, such as vancomycin, linezolid, rifampicin, and fusidic acid (2,6).

The molecular epidemiology of MRSA can be studied using different typing methods, such as staphylococcal cassette chromosomal mec (SCCmec) typing, multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). SCCmec typing classifies MRSA based on the combinations of mec and ccr gene complexes (7). PFGE is useful for local epidemiological studies and tracing of the source of outbreaks, especially when there are several concurrent epidemic episodes. In addition, MLST has also been used as a highly discriminatory method for molecular evolutionary studies on MRSA (8). In brief, the 450-bp internal fragments of seven housekeeping genes are sequenced and blasted against a database in www.mlst.net. A sequence type (ST) is then assigned and subjected to an e-burst logarithm (http://eburst.mlst.net/v3/mlst_datasets/) to group different STs into different clonal complexes (CCs), at which microbial evolution can be studied. Major CCs related to HA-MRSA are CC5, CC8, CC22, CC30, and CC45, while CCs related to CA-MRSA are CC1, CC8, CC30, CC59, and CC80 linkages (9). In Malaysia, Lim et al. and Sit et al. had conducted molecular epidemiology studies on MRSA isolated from the same teaching hospital from 2003-2008 and 2011-2012, respectively. Both studies concurred that the ST239-MRSA-III clone was the predominant clone circulating in that hospital. ST239-MRSA-III was also reported as the predominant clone in different teaching hospitals in Terengganu and Kuala Lumpur (10,11).

In addition to changing molecular characteristics, a decrease in vancomycin susceptibility had also been observed among MRSA clinical isolates collected from multicenters in Malaysia (5). It is a great concern as it might soon compromise vancomycin as the main armamentarium against MRSA infections. However, there has been limited correlation between clinical data and changing trends of MRSA isolates over the years.
In this study, we aimed to characterize MRSA clinical isolates from a tertiary teaching hospital within a 2-year period based on their molecular and phenotypic characteristics. The data were then correlated with clinical data retrieved in this study.

**MATERIALS AND METHODS**

**Hospital setting and ethical approval:** The study was conducted at the University Malaya Medical Centre (UMMC), a 980-bed tertiary teaching hospital in Kuala Lumpur, which consists of different units, such as surgical, pediatric, medical, obstetrics and gynecology, intensive care units, psychiatric units, and orthopedics units. Ethics approval was obtained from the Medical Ethic Committee of UMMC on June 7th, 2014 (MEC ID: 20145-168) and conformed to the principles embodied in the Declaration of Helsinki.

**Bacterial isolates and patients demographic:** Ninety-nine non-duplicated isolates (n = 99) were previously identified as MRSA by the Diagnostic laboratory of UMMC. MRSA isolates were stocked once they were detected from clinical samples obtained from patients. The isolates were isolated from adult patients aged ≥16 years and isolated from different sterile sites such as blood, cerebrospinal fluid (CSF), subcutaneous hip fluid, bone, and pleural effusion from January 2014 to December 2015. The MRSA strains were re-confirmed with PCR targeting the femA gene prior to commencing the bench work.

Patients’ data, such as age, race, underlying diseases, mode of acquisition of MRSA, history of previous hospitalization, antibiotics exposure in the past 90 days, and nasal colonization were retrieved for correlation analysis. MRSA infections of patients were epidemiologically classified into three categories: healthcare-associated-hospital-onset (HA-MRSA-HO) infection, healthcare-associated-community-onset (HA-MRSA-CO) infection, and community-associated (CA-MRSA) infection based on data collected from medical records and the database of Infection Control’s Department. An HA-MRSA-HO infection refers to positive cultures obtained from patients in >48 h after admission or from those who had undergone surgery in the previous month. An HA-MRSA-CO infection refers to positive cultures isolated from patients in <48 h of admission and who had had contact with healthcare system within the previous three months, those who came from a nursing home, or those on regular hemodialysis. A CA-MRSA infection refers to positive cultures obtained from patients in <48 h after hospital admission, with the absence of any of the previously stated healthcare risk factors.

**Characterization of MRSA SCCmec:** SCCmec typing was performed on mecA positive MRSA isolates using primers and modified multiplex PCR conditions as described by Milheiro et al. (12) and Hisata et al. (13). PCR was performed in a SimpliAmp thermocycler (Thermo Fisher Scientific, MA, USA), Gel electrophoresis was performed by applying 3 µL of PCR products to a 2% agarose gel. Gels were run for 60 min at 120 V in 0.5x TBE (tris-borate-EDTA) buffer. A DNA molecular weight marker, 100 bp DNA ladder (Promega, WI, USA) was used as a size standard.

Gels were visualized by UV illumination after staining with SYBE® Safe DNA Gel Stain (Life Technologies, CA, USA). The following reference isolates were used: NCTC 10442 for SCCmec Type I, N315 for SCCmec Type II, 85/2082 for SCCmec Type III, NCTC 4744 for SCCmec Type IVa, JCSC 2172 for SCCmec Type IVb, MR108 for SCCmec Type IvC, JCSC 4469 for SCCmec Type IvD, and WIS for SCCmec Type V. These reference strains were kindly provided by K.L. Thong, University of Malaya. In order to confirm the reproducibility of laboratory results, PCR was performed at least twice for each isolate.

**Agr genotyping and virulence genes detection:** Multiplex PCR amplification was performed on MRSA isolates to group them into different agr types using specific primers and conditions (14). Detection of 20 virulence genes (sea, seb, sec, sed, see, seh, sei, sej, eta, etb, etd, tst, fib, fnbA, fnbB, hlg, icaA, sdrE, and pvl) was performed based on the primers described previously (15-20). Previously reported positive isolates for sea, sec, see, seg, sei, etd, and hlg were kindly provided by K.L. Thong, University of Malaya. Representative amplicons of seh, sej, icaA, fib, fnbB, and pvl were purified and sequenced to validate their identities. PCR was performed at least twice to confirm their reproducibility.

**MLST:** All clinical isolates were characterized by MLST as described by Enright et al (12) in order to determine the ST of each MRSA isolate. Specific gene fragments of arcC, aroE, glpF, gmk, pta, tpi, and yqiL housekeeping loci were amplified through PCR. All amplicons were compared and blasted against the MLST database (http://www.mlst.net) to determine the allelic profiles and ST. MRSA isolates were then grouped into different CCs using E-burst algorithm (http://eburst.mlst.net/v3/mlst_datasets/).

**PFGE:** PFGE was performed by Smal macrorestriction of genomic DNA, followed by electrophoresis according to a protocol described by Sit et al. (21). The gels were stained with GelRed system (10,000x in water; Biotium, CA, USA) for 30 min and viewed under UV illumination. The Bioinformatics software, version 6.6 (AppliedMaths, Belgium), was used for similarity analyses and calculation of Dice coefficient. A dendrogram was generated based on UPGMA (unweighted pair group method using arithmetic averages) with band position tolerance and optimization of 80%. A similarity coefficient of 80% was chosen for the determination of genetic relatedness of MRSA isolates by grouping used as the molecular size standard.

**Antibiotic susceptibility profiles:** Vancomycin susceptibility data of the isolates were obtained from a microbiology diagnostic laboratory. The minimal inhibitory concentration (MIC) of vancomycin was confirmed using a Vitek 2 system (bioMérieux, Craponne, France) and interpreted based on Clinical and Laboratory Standards Institute (CLSI) guidelines (22). Susceptibility data of the isolates to antibiotics, such as ampicillin-sulbactam, erythromycin, gentamicin, cefoxitin, rifampicin, fusidic acid, ciprofloxacin, clindamycin, piperacillin-tazobactam, and penicillin was retrieved from the diagnostic laboratory database.

**Statistical analysis:** The genotypic, phenotypic, and
clinical data collected were analyzed using SPSS version 22 (SPSS, Chicago, IL, USA). Dichotomous variables were analyzed by the chi-squared or Fisher’s exact tests wherever appropriate. A p-value <0.05 was considered statistically significant.

RESULTS

Clinical background: A total of 99 MRSA isolates were collected from different sterile sites including blood (n = 77, 77.77%), CSF (n = 3, 3.03%), pleural fluid (n = 4, 4.04%), subcutaneous hip fluid (n = 1, 1.01%), and bones (n = 14, 14.14%). The median age of studied patients was 61 years (range 17-94). Cases of hypertension (p = 0.035) and dyslipidemia and obesity (p = 0.046) increased significantly in 2015 when compared to the previous year (2014). Moreover, a significant increase in pre-exposure to quinolone (p = 0.010) was observed in 2015. Statistically significant values were obtained for gender and vancomycin MIC for MRSA in both years. When a descriptive analysis was performed, SCCmec element was significantly associated with pvl gene presence (p = 0.000) in MRSA isolates. The demographics of patients and characteristics of isolates by year are listed in Table 1.

SCCmec typing: Three SCCmec types, III, IV, and V were detected among the isolates. The predominant SCCmec type was SCCmec type IV (n = 70, 70.71%).

| Characteristics          | 2014 (n = 46) N (%) | 2015 (n = 53) N (%) | All subjects (n = 99) | P-value |
|--------------------------|---------------------|---------------------|-----------------------|---------|
| Age                      |                     |                     |                       |         |
| ≤ 50 years old           | 14 (51.9)           | 13 (48.1)           | 27                    | 0.651   |
| > 50 years old           | 32 (44.4)           | 40 (55.6)           | 72                    |         |
| Sex                      |                     |                     |                       | 0.045²  |
| Male                     | 21 (37.5)           | 35 (62.5)           | 56                    |         |
| Female                   | 25 (58.1)           | 18 (41.9)           | 43                    |         |
| Ethnicity                |                     |                     |                       | 0.620   |
| Malay                    | 16 (53.3)           | 14 (46.7)           | 30                    |         |
| Chinese                  | 15 (45.5)           | 18 (54.5)           | 33                    |         |
| Indian                   | 14 (45.2)           | 17 (54.8)           | 31                    |         |
| Others                   | 1 (20.0)            | 4 (80.0)            | 5                     |         |
| Onset of infection       |                     |                     |                       | 0.669   |
| HA                       | 28 (43.8)           | 36 (56.3)           | 64                    |         |
| HA-MRSA-HO              | 14 (53.8)           | 12 (46.2)           | 26                    |         |
| CA                       | 4 (44.4)            | 5 (55.6)            | 9                     |         |
| Comorbidities            |                     |                     |                       |         |
| Diabetes mellitus        | 26 (45.6)           | 31 (54.4)           | 57                    | 1.000   |
| Hypertension             | 25 (38.5)           | 40 (61.5)           | 65                    | 0.035   |
| Moderate to chronic kidney disease | 22 (47.8) | 24 (52.2) | 46 | 0.842 |
| Dyslipidemia & obesity   |                     |                     |                       |         |
| CVA³                    | 3 (20.0)            | 12 (80.0)           | 15                    | 0.046   |
| Malignancy               | 7 (53.8)            | 6 (46.2)            | 13                    | 0.767   |
| Respiratory disease²     | 7 (70.0)            | 3 (30.0)            | 10                    | 0.181   |
| Cardiovascular disease³  | 1 (14.3)            | 6 (85.7)            | 7                     | 0.118   |
|                          | 6 (31.6)            | 13 (68.4)           | 19                    | 0.202   |
| Previous hospitalization |                     |                     |                       | 0.526   |
| < 3 months ago           | 32 (49.2)           | 33 (50.8)           | 65                    |         |
| 3-6 months               | 2 (66.7)            | 1 (33.3)            | 3                     |         |
| 6-12 months              | 2 (100.0)           | 0 (0.0)             | 2                     |         |
| No or more than 1 year   | 6 (33.3)            | 12 (66.7)           | 18                    |         |
| None or non-applicable   | 4 (36.4)            | 7 (63.6)            | 11                    |         |
| Previous antibiotics exposure |                   |                     |                       |         |
| Any antibiotics          | 33 (44.6)           | 41 (55.4)           | 74                    | 0.499   |
| Any penicillin           | 15 (39.5)           | 23 (60.5)           | 38                    | 0.305   |
| Any cephalosporin        | 18 (39.1)           | 28 (60.9)           | 46                    | 0.226   |
| Any macrolide            | 1 (12.5)            | 7 (87.5)            | 8                     | 0.065   |
| Any quinolone            | 2 (14.3)            | 12 (85.7)           | 14                    | 0.010   |
| Any carbapenem           | 8 (42.1)            | 11 (57.9)           | 19                    | 0.800   |
| Metronidazole            | 5 (35.7)            | 9 (64.3)            | 14                    | 0.407   |
| Tazocin                  | 12 (41.4)           | 17 (58.6)           | 29                    | 0.514   |
| Vancomycin               | 3 (30.0)            | 7 (70.0)            | 10                    | 0.331   |
| SCCmec types             |                     |                     |                       | 0.595   |
| III                      | 15 (57.7)           | 11 (42.3)           | 26                    |         |
| IV                       |                     |                     |                       |         |
| IVa                      | 5 (50.0)            | 5 (50.0)            | 10                    |         |
| IVc                      | 2 (40.0)            | 3 (60.0)            | 5                     |         |
| Novel subtypes           | 22 (40.0)           | 33 (60.0)           | 55                    |         |
| V                        | 2 (66.7)            | 1 (33.3)            | 3                     |         |
| PVL gene⁴               | 2 (33.3)            | 4 (66.7)            | 6                     | 0.683   |
These isolates were further subtyped into IVa (n = 10, 10.10) and IVc (n = 5, 5.10). Fifty-five SCCmec type IV isolates were subtype untypable. SCCmec type III was detected in 26 (26.3\%) isolates, while SCCmec type V was detected in 3 (3.0\%) isolates.

Agr genotyping and virulence genes detection:
Ninety-eight MRSA isolates showed 3 agr genotypes: agr type I (91.9\%), agr type II (4.0\%), and agr type III (3.0\%). Agr type IV was not detected in the isolates. One strain did not belong to any of the agr groups.

All agr type II strains were isolated from blood but belonged to different modes of acquisition, whereas agr type III strains were isolated from different sites and belonged to HA-MRSA-HO infection. The most prevalent virulence genes were hemolysin (hlg) (100\%) and intracellular adhesion (icaA) (91.9\%). Isolates were positive for adhesion genes such as fibrinogen-binding protein precursor (fib) (44.4\%) and fibrinogen-binding protein B (fnbB) (28.3\%). Putative adhesion (sdrE) and fibrinogen binding protein A (fnbA) were not amplified.

Table 2. Prevalence of genes encoding virulence determinants in 99 MRSA isolates in 2014 and 2015

| Gene      | No. of MRSA positive strains (n = 99) | p value | Gene combination                  | No. of MRSA positive strains (n = 99) |
|-----------|--------------------------------------|---------|-----------------------------------|--------------------------------------|
|           | 2014  | 2015  |                                  | 2014  | 2015  |
| icaA\(^1\) | 36    | 55    | 0.020\(^2\)                      | 1     | 0     |
| fnbB      | 14    | 14    | 0.408                            | 6     | 3     |
| fib       | 22    | 22    | 0.238                            | 1     | 0     |
| hlg       | 43    | 56    | –                                | 1     | 1     |
| sea       | 12    | 11    | 0.334                            | 1     | 0     |
| sec       | 7     | 4     | 0.201                            | 0     | 1     |
| see       | 0     | 1     | 1.000                            | 16    | 31    |
| seg       | 25    | 35    | 0.660                            | 9     | 10    |
| seh       | 1     | 0     | 0.434                            | 0     | 1     |
| sei       | 25    | 35    | 0.660                            | 0     | 1     |
| sei       | 1     | 2     | 1.000                            | 35    | 48    |
| pvl       | 1     | 5     | 0.229                            | (81.4\%) | (85.7\%) |

\(^1\) icaA, intracellular adhesion A; fnbB, fibrinogen-binding protein B; fib, fibrinogen-binding protein precursor; hlg, hemolysin; sea, enterotoxin A; sec, enterotoxin C; see, enterotoxin E; seg, enterotoxin G; seh, enterotoxin H; sei, enterotoxin I; sej, enterotoxin J; pvl, panton-valentine leukocidin.

\(^2\) Bold text indicates P-value < 0.05, which was considered to be statistically significant.

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ST22-MRSA-IV in a Teaching Hospital

Table 1. Demographics of patients and characteristics of isolates by year (continued)

| CC/ST\(^5\) | 2014 | 2015 | p value | CC/ST\(^5\) | 2014 | 2015 | p value |
|-------------|------|------|---------|-------------|------|------|---------|
| ST1         | 1 (100.0) | 0 (0.0) | 1 | ST1         | 1 (100.0) | 0 (0.0) | 1 |
| ST769       | 0 (0.0) | 1 (100.0) | 1 | ST772       | 1 (100.0) | 0 (0.0) | 1 |
| CC5         | 1 (50.0) | 1 (50.0) | 2 | ST1178      | 0 (0.0) | 1 (100.0) | 1 |
| ST6         | 2 (50.0) | 2 (50.0) | 4 | ST8         | 0 (0.0) | 2 (100.0) | 2 |
| ST8         | 0 (0.0) | 2 (100.0) | 2 | ST239       | 15 (57.7) | 11 (42.3) | 26 |
| CC22        | 22 (40.0) | 33 (60.0) | 55 | CC45        | 2 (100.0) | 0 (0.0) | 2 |
| CC45        | 2 (100.0) | 0 (0.0) | 1 | CC88        | 1 (50.0) | 1 (50.0) | 2 |
| CC88        | 0 (100.0) | 0 (0.0) | 1 | CC1047      | 1 (50.0) | 1 (100.0) | 1 |
| Nasal colonization | 4 (36.4) | 7 (63.6) | 11 | Vancomycin MIC\(^6\) | 40 (43.5) | 52 (56.5) | 92 |
| <1.5ug/ml  | 6 (85.7) | 1 (14.3) | 7 | >1.5ug/ml  | 0.047 | 0.047 | 0.047 |

\(^5\) CC, clonal complex.

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\(^1\) CVA, cerebrovascular accident.

\(^2\) Respiratory diseases includes: chronic obstructive pulmonary disease (COPD), pneumonia, pulmonary embolism, acute respiratory distress syndrome.

\(^3\) Cardiovascular diseases includes: congestive cardiac failure, mitral valve regurgitation, acute coronary syndrome, ischaemic heart disease.

\(^4\) PVL, panton-valentine leucocidin.

\(^6\) MIC, minimal inhibitory concentration.

\(^7\) Fisher’s exact test done for categorical variables, bold text indicates P-value < 0.05, which was considered to be statistically significant.
Fig. 1. Dendrogram of MRSA PFGE patterns generated by UPGMA clustering method using Dice coefficient. The dotted line indicates an arbitrary 80% similarity demarcation. C, cluster; SCCmec, staphylococcal cassette chromosome mec; PVL, panton-valentine leukocidin; ST, sequence type; MRSA, methicillin-resistant Staphylococcus aureus.
In the detection of staphylococcal enterotoxins (SEs), a total of 83 (83.8%) strains were positive for at least one SEs. No enterotoxins (seb, sed, tst) or exfoliative toxins (eta, etb, etd) were detected. The Panton-Valentine leukocidin (pvl) gene was detected in 6 (6.1%) isolates. It was distributed among 4 SCCmec type IVa isolates, 1 SCCmec type IVc isolate, and 1 SCCmec type V isolate. The prevalence of genes encoding virulence factors was observed in 70.7% of the isolates, ST22 was observed in 55 isolates, followed by ST6 in 4 isolates, ST188, ST45, ST5, and ST8 each in 2 isolates, and ST1178, ST88, and ST769 each in 1 isolate. Based on our findings, 64.29% of SCCmec type IV isolates showed diverse STs:

| STR Lid | 2014 | 2015 | Total | P-value |
|--------|------|------|-------|---------|
| ST22   | 55   | 55.6 | 110   |         |
| ST6    | 26   | 26.3 | 52    |         |
| ST1    | 4    | 4.0  | 8     |         |
| ST188  | 2    | 2.0  | 4     |         |
| ST45   | 2    | 2.0  | 4     |         |
| ST5    | 2    | 2.0  | 4     |         |
| ST8    | 2    | 2.0  | 4     |         |
| ST1    | 1    | 1.0  | 2     |         |
| ST772  | 1    | 1.0  | 2     |         |
| ST1178 | 1    | 1.0  | 2     |         |
| ST88   | 1    | 1.0  | 2     |         |
| ST769  | 1    | 1.0  | 2     |         |
| MRSA   | 9    | 9.0  | 18    |         |
| SCCmec | 4    | 4.0  | 8     |         |
| PVL    | 6    | 6.1  | 12    |         |

In our present study, MRSA isolates collected from hospitalized patients were still susceptible to vancomycin, at which the MICs of all clinical isolates were within the range of <0.5 µg/mL and 2 µg/mL. MRSA isolates remained susceptible to rifampicin, fusidic acid, and gentamicin, which account for 4.0%, 23.2%, and 28.3% of resistance rates, respectively. Other than that, resistance towards ampicillin-sulbactam (82.8%), erythromycin (85.9%), ciprofloxacin (86.9%), clindamycin (84.8%), and piperacillin-tazobactam (79.8%) were high in the tested isolates. The resistance patterns of MRSA isolates in 2014 and 2015 are shown in Table 3. A statistical difference was observed for gentamicin resistance (p = 0.029) in both years.

**DISCUSSION**

To date, 13 SCCmec types have been identified worldwide, however, only 5 were reported in Malaysia. In this study, all 99 strains were successfully typed and 3 SCCmec types: II, IV, and V were detected. SCCmec type IV was the most predominant SCCmec type and was detected in 70.7% (n = 70) of the clinical isolates. These SCCmec type IV isolates showed diverse STs: ST22 was observed in 55 isolates, followed by ST6 in 4 isolates, ST1178, ST88, and ST769 each in 2 isolates, and ST45 in 1 isolate. Based on our findings, 64.29% of SCCmec type IV was HA-
The majority of MRSA isolates in this 2-year study period belonged to the pandemic clone SCCmec IV-ST22 and most of them had pulsortype 28 and 29. Combined analyses based on PFGE, MLST, and SCCmec types (ST22-MRSA-IV) showed that most of the MRSA isolates were clonally related, even though they were detected from different specimens isolated from different patients in both years. This suggests the possibility of circulation and persistence of specific clones in this tertiary teaching hospital during the study period. In addition, the presence of clonally related isolates from different patients during the same period of time and with the same mode of acquisition of MRSA suggested potential clonal spread of MRSA clones within wards, between different wards, and within healthcare settings; the most likely mode of transmission was by direct contact. The combined analyses used in this study illustrated the potential clonal relatedness of the strains, however, the genetic linkage could be further elucidated if high resolution tools, such as whole genome sequencing (WGS) were being used.

In the process of subtyping, 55 SCCmec type IV isolates were untypeable by SCCmec IV subtyping; they might belong to novel SCCmec subtypes.

In addition, the majority of the MRSA isolates belonged to ST22-MRSA-IV. This clone was first reported in UK and later emerged in Malaysia (5, 23). More recently, this clone has superseded ST239-MRSA-III, which was previously reported as the predominant epidemic HA-MRSA clone in some of the hospitals in Malaysia (5,11,21,23). This might be due to the smaller size of SCCmec type IV elements and different virulence factors harbored by this clone, which may also promote pathogenicity and persistence (11).

In our study, the pvl gene was present in both SCCmec type IV and V isolates. All the pvl positive isolates were HA-MRSA-HO, which were clinically presented as bacteremia (3 patients), catheter-related bloodstream infection (CRBSI) (1 patient), surgical site infection (SSI) and implant-related infection (IRI) (1 patient), and skin and soft tissue infection (SSTI), surgical site infection (SSI) and bone infection (1 patient). These findings indicated the emergence of HA-MRSA-HO infections caused by SCCmec types IV and V, and posed a significant threat to the patients. This finding concurred with Sun et al. and Valle et al., who reported an increasing presence of pvl virulence factor in a hospital setting over the recent years (24,25).

The majority of the strains (91%) were of agr type I and this was consistent with previous findings (11.26). Overall, 83% of isolates harbored at least one SEs gene and the percentage of 2015 strains (85.7%) that harbored these genes was higher than 2014 strains (81.4%). This might be attributed to horizontal transfer of genes among the strains through plasmids, SCCmec, pathogenicity islands, and prophages (27). Seg and sei were the most common SEs genes found in MRSA isolates in 2014 and 2015. As seg and sei were located in the same egr operon (28), the simultaneous presence of seg + sei genes in the isolates was common in the tested isolates. A significant increase of the icaA gene in 2015 strains is worrying as co-existence of biofilm-associated genes with SEs genes is normally contributing to antimicrobial and host immune system resistance (29).

Most of seg + sei (n = 44) and sec + seg + sei gene combinations (n = 9) were observed in ST22-MRSA-IV isolates. This finding suggests penetration of diverse ST-22-MRSA-IV isolates in our center. Enterotoxin H was present in 1 ST1 isolate, resulting in HA-MRSA-HO infection. Meanwhile, sea was detected in diversified STs (ST239, ST6, ST1, ST22, and ST772), which was similarly reported previously (11.26). This is the first report on the emergence of enterotoxin J in MRSA isolates in this center. It was isolated from different sites, causing HA-MRSA-HO and HA-MRSA-IV infections, suggesting dissemination of sei in the healthcare environment. On the other hand, different types of enterotoxin genes were harbored by strains with similar pulsortypes within the same cluster.

MRSA-HO, followed by 27.14% HA-MRSA-CO, and 8.57% CA-MRSA. A similar finding was reported by Ahmad et al. (5), where a higher percentage of ST22-MRSA-IV isolates belonged to HA-MRSA. All SCCmec type III isolates were ST239. In contrast, SCCmec type V isolates were comprised of ST1, ST772, and ST3547. In the process of subtyping, 55 SCCmec type IV isolates were untypeable by SCCmec IV subtyping; they might belong to novel SCCmec subtypes.

In the study, the pvl gene was present in both SCCmec type IV and V isolates. All the pvl positive isolates were HA-MRSA-HO, which were clinically presented as bacteremia (3 patients), catheter-related bloodstream infection (CRBSI) (1 patient), surgical site infection (SSI) and implant-related infection (IRI) (1 patient), and skin and soft tissue infection (SSTI), surgical site infection (SSI) and bone infection (1 patient). These findings indicated the emergence of HA-MRSA-HO infections caused by SCCmec types IV and V, and posed a significant threat to the patients. This finding concurred with Sun et al. and Valle et al., who reported an increasing presence of pvl virulence factor in a hospital setting over the recent years (24,25).

The majority of the strains (91%) were of agr type I and this was consistent with previous findings (11.26). Overall, 83% of isolates harbored at least one SEs gene and the percentage of 2015 strains (85.7%) that harbored these genes was higher than 2014 strains (81.4%). This might be attributed to horizontal transfer of genes among the strains through plasmids, SCCmec, pathogenicity islands, and prophages (27). Seg and sei were the most common SEs genes found in MRSA isolates in 2014 and 2015. As seg and sei were located in the same egr operon (28), the simultaneous presence of seg + sei genes in the isolates was common in the tested isolates. A significant increase of the icaA gene in 2015 strains is worrying as co-existence of biofilm-associated genes with SEs genes is normally contributing to antimicrobial and host immune system resistance (29).

Most of seg + sei (n = 44) and sec + seg + sei gene combinations (n = 9) were observed in ST22-MRSA-IV isolates. This finding suggests penetration of diverse ST-22-MRSA-IV isolates in our center. Enterotoxin H was present in 1 ST1 isolate, resulting in HA-MRSA-HO infection. Meanwhile, sea was detected in diversified STs (ST239, ST6, ST1, ST22, and ST772), which was similarly reported previously (11.26). This is the first report on the emergence of enterotoxin J in MRSA isolates in this center. It was isolated from different sites, causing HA-MRSA-HO and HA-MRSA-CO infections, suggesting dissemination of sei in the healthcare environment. On the other hand, different types of enterotoxin genes were harbored by strains with similar pulsortypes within the same cluster.
resistant *Staphylococcus aureus* (MRSA): global epidemiology and harmonisation of typing methods. Int J Antimicrob Agents. 2012;39:273-82.

4. Rohani MY, Raudzah A, Lau MG, et al. Susceptibility pattern of *Staphylococcus aureus* isolated in Malaysian hospitals. Int J Antimicrob Agents. 2000;13:209-13.

5. Ahmad N, Ruzan IN, Abd Ghanai MK, et al. Characteristics of community- and hospital-acquired meticillin-resistant *Staphylococcus aureus* strains carrying SCCmec type IV isolated in Malaysia. J Med Microbiol. 2009;58:1213-8.

6. Matthew TGH, Feil EJ, Lindsay JA, et al. Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A. 2004;97:86-91.

7. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of Staphylococcal cassette chromosome mec (SCCmec): guidelines for reporting novel SCCmec elements. Antimicrob Agents Chemother. 2009;53:4961-7.

8. Enright MC, Day NP, Davies CE, et al. Multilocus sequence typing for characterization of methicillin-resistant and meticillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 2003:38:1008-15.

9. David MZ, Daum RS. Community-associated meticillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010;23:616-87.

10. Lim KT, Hanifah YA, Mohd Yusof MY, et al. Investigation of toxin genes among meticillin-resistant *Staphylococcus aureus* strains isolated from the University Hospital, Malaysia. J Microbiol Immunol Infect. 2013;46:224-33.

11. Hu DL, Omoe K, Inoue F, et al. Comparative prevalence of superantigenic toxin genes in meticillin-resistant and meticillin-susceptible *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. J Microbiol Immunol Infect. 2013;46:682-90.

12. Valle DL Jr, Paclibare PA, Cabrera EC, et al. Molecular and phenotypic characterization of meticillin-resistant *Staphylococcus aureus* isolates from a tertiary hospital in the Philippines. Trop Med Health. 2016;44:3-4.

13. Lim KT, Hanifah YA, Mohd Yusof MY, et al. Investigation of toxin genes andamp;#39;genes among meticillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. Tropical biomedicine. 2012;29:212-9.

14. Plata K, Rosato AE, Wegrzyn G. *Staphylococcus aureus* as an infectious agent: overview of biochemistry and molecular genetics of its pathogenicity. Acta Biochim Pol. 2009;56:597-612.

15. Jarraud S, Mougel C, Thioulouse J, et al. Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. Infect Immun. 2002;70:631-41.

16. Lina G, Piémont Y, Gadal-Garnot F, et al. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clin Infect Dis. 1999;29:1128-32.

17. Tristan A, Ying L, Bes M, et al. Use of multiplex PCR to identify *Staphylococcus aureus* adhesins involved in human hematogenous infections. J Clin Microbiol. 2003;41:4465-7.

18. Yamaguchi T, Nishifuji K, Sasaki M, et al. Identification of the *Staphylococcus aureus* etd pathogenicity island which encodes a novel exfoliative toxin, ETDT, and EDIN-B. Infect Immun. 2002;70:5835-45.