Jpn. J. Infect. Dis., 72, 228–236, 2019

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

Wen Kiong Niek[1], Cindy Shuan Ju Teh[1], Nuryana Idris[1], Kwai Lin Thong[2], and Sasheela Ponnampalavanar[3]*

[1]Department of Medical Microbiology, [3]Department of Medicine, Faculty of Medicine, and [2]Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

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* (SCC*mec*) 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 SCC*mec* types (III 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 $\leq$ 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* (SCC*mec*) typing, multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). SCC*mec* 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.

Received January 27, 2019. Accepted February 1, 2019.
J-STAGE Advance Publication February 28, 2019.
DOI: 10.7883/yoken.JJID.2018.289
*Corresponding author: Mailing address: Department of Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia. Tel: +6012-203 5566, E-mail: sheela@ummc.edu.my.

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 have 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: SCC*mec* typing was performed on *mec*A positive MRSA isolates using primers and modified multiplex PCR conditions as described by Milheirico 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 SCC*mec* Type I, N315 for SCC*mec* Type II, 85/2082 for SCC*mec* Type III, NCTC 4744 for SCC*mec* Type IVa, JCSC 2172 for SCC*mec* Type IVb, MR108 for SCC*mec* Type IVc, JCSC 4469 for SCC*mec* Type IVd, and WIS for SCC*mec* 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, seg, 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 *SmaI* 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 transillumination. The Bionumerics 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 at 1.5% and 1.0%, respectively. 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, SCC*mec* 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.

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

Table 1. Demographics of patients and characteristics of isolates by year

Characteristics	2014 (*n* = 46) *N* (%)	2015 (*n* = 53) *N* (%)	All subjects (*n* = 99)	*P*-value
Age				0.651
≤ 50 years old	14 (51.9)	13 (48.1)	27	
> 50 years old	32 (44.4)	40 (55.6)	72	
Sex				**0.045**[7]
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				
HA-MRSA-HO	28 (43.8)	36 (56.3)	64	
HA-MRSA-CO	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				
CVA[1]	3 (20.0)	12 (80.0)	15	**0.046**
Malignancy	7 (53.8)	6 (46.2)	13	0.767
Respiratory disease[2]	7 (70.0)	3 (30.0)	10	0.181
Cardiovascular disease[3]	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 ago	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
SCC*mec* 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 gene[4]	2 (33.3)	4 (66.7)	6	0.683

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

CC/ST[5]				0.394
CC1				
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				
ST5	1 (50.0)	1 (50.0)	2	
ST1178	0 (0.0)	1 (100.0)	1	
CC6				
ST6	2 (50.0)	2 (50.0)	4	
CC8				
ST8	0 (0.0)	2 (100.0)	2	
ST239	15 (57.7)	11 (42.3)	26	
CC22				
ST22	22 (40.0)	33 (60.0)	55	
CC45				
ST45	2 (100.0)	0 (0.0)	2	
CC88				
ST88	1 (100.0)	0 (0.0)	1	
CC188				
ST188	1 (50.0)	1 (50.0)	2	
CC1047				
ST3547	0 (0.0)	1 (100.0)	1	
Nasal colonization	4 (36.4)	7 (63.6)	11	0.658
Vancomycin MIC[6]				**0.047**
≤ 1.5μg/ml	40 (43.5)	52 (56.5)	92	
> 1.5μg/ml	6 (85.7)	1 (14.3)	7	

[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.
[5]: CC, clonal complex.
[6]: MIC, minimal inhibitory concentration.
[7]: Fisher's exact text done for categorical variables, bold text indicates *P*-value < 0.05, which was considered to be statistically significant.

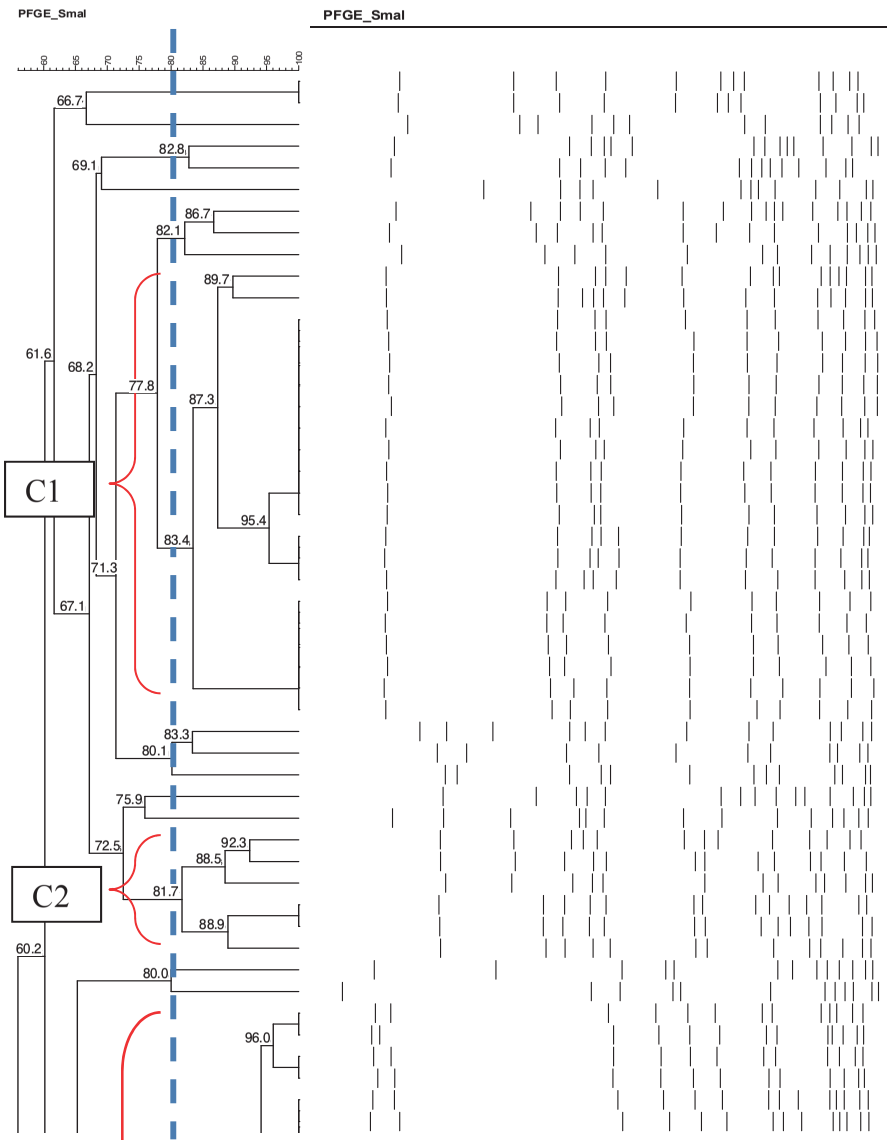
These isolates were further subtyped into IVa (n = 10, 10.10%) and IVc (n = 5, 5.10%). Fifty-five SCC*mec* type IV isolates were subtype untypable. SCC*mec* type III was detected in 26 (26.3%) isolates, while SCC*mec* type V was detected in 3 (3.0%) isolates.

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 acquistion, 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]	*sea + sec + seg + sei*	1	0
fnbB	14	14	0.408	*sec + seg + sei*	6	3
fib	22	22	0.238	*seg + sei + sej*	1	0
hlg	43	56	–	*sea + seg + sei*	1	1
sea	12	11	0.334	*sea + seh*	1	0
sec	7	4	0.201	*see + sej*	0	1
see	0	1	1.000	*seg + sei*	16	31
seg	25	35	0.660	*sea*	9	10
seh	1	0	0.434	*sec*	0	1
sei	25	35	0.660	*sej*	0	1
sej	1	2	1.000	Total	35 (81.4%)	48 (85.7%)
pvl	1	5	0.229			

[1]: *icaA,* intacellular 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*, enterotixin I; *sej*, enterotoxin J; *pvl*, panton-valentine leucocidin.
[2]: Bold text indicates *P*-value < 0.05, which was considered to be statistically significant.



Strain ID	Specimens	SCCmec	PVL	ST	MRSA acquisition	Pulsotype
ST/1511-11	BLOOD	IVc	-	8	CA-MRSA	1
ST/1512-26	BLOOD	IVc	-	8	HA-MRSA-HO	1
ST/1401-2	BLOOD	III	-	239	HA-MRSA-CO	2
ST/1402-15	BLOOD	III	-	239	HA-MRSA-CO	3
ST/1508-16	BLOOD	III	-	239	HA-MRSA-HO	4
ST/1407-16	BLOOD	V	+	772	CA-MRSA	5
ST/1404-17	BLOOD	III	-	239	CA-MRSA	6
ST/1403-23	BLOOD	III	-	239	HA-MRSA-HO	7
ST/1510-3	BONE	IVa	+	769	HA-MRSA-HO	8
ST/1401-15	BLOOD	III	-	239	CA-MRSA	9
ST/1406-7	PF	III	-	239	HA-MRSA-HO	10
ST/1508-3	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1508-2	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1508-1	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1508-28	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1509-1	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1507-27	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1408-3	BLOOD	III	-	239	HA-MRSA-CO	11
ST/1505-22	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1506-10	BLOOD	III	-	239	HA-MRSA-HO	11
ST/1405-6	PF	III	-	239	HA-MRSA-HO	11
ST/1407-9	BLOOD	III	-	239	HA-MRSA-CO	12
ST/1406-18	BLOOD	III	-	239	HA-MRSA-CO	12
ST/1501-7	BLOOD	III	-	239	HA-MRSA-HO	12
ST/1408-15	BLOOD	III	-	239	HA-MRSA-HO	13
ST/1404-5	BLOOD	III	-	239	HA-MRSA-HO	13
ST/1403-8	BLOOD	III	-	239	HA-MRSA-CO	13
ST/1403-3	BLOOD	III	-	239	HA-MRSA-HO	13
ST/1402-7	BLOOD	III	-	239	HA-MRSA-HO	13
ST/1502-6	BONE	III	-	239	HA-MRSA-HO	13
ST/1408-37	BLOOD	IVc	-	5	HA-MRSA-HO	14
ST/1506-7	BLOOD	IVc	+	5	HA-MRSA-HO	15
ST/1503-17	BLOOD	IV	-	1178	HA-MRSA-CO	16
ST/1502-3	BLOOD	V	-	3547	HA-MRSA-CO	17
ST/1512-19	BLOOD	IVa	-	188	HA-MRSA-HO	18
ST/1410-17	BONE	IVa	-	188	HA-MRSA-HO	19
ST/1411-6	PF	IVa	-	6	HA-MRSA-CO	20
ST/1402-2	BLOOD	V	-	1	HA-MRSA-HO	21
ST/1501-44	BLOOD	IVa	-	6	HA-MRSA-HO	22
ST/1411-16	BLOOD	IVa	-	6	HA-MRSA-HO	22
ST/1512-22	BLOOD	IVa	+	6	HA-MRSA-HO	23
ST/1406-25	BLOOD	IVc	-	45	HA-MRSA-CO	24
ST/1402-30	BLOOD	IVa	-	45	HA-MRSA-CO	25
ST/1401-9	BLOOD	IV	-	22	HA-MRSA-HO	26
ST/1508-8	BLOOD	IV	-	22	CA-MRSA	26
ST/1407-14	BLOOD	IV	-	22	HA-MRSA-CO	27
ST/1504-6	BLOOD	IV	-	22	HA-MRSA-CO	27
ST/1405-13	BLOOD	IV	-	22	HA-MRSA-CO	28
ST/1403-4	BLOOD	IV	-	22	HA-MRSA-HO	28

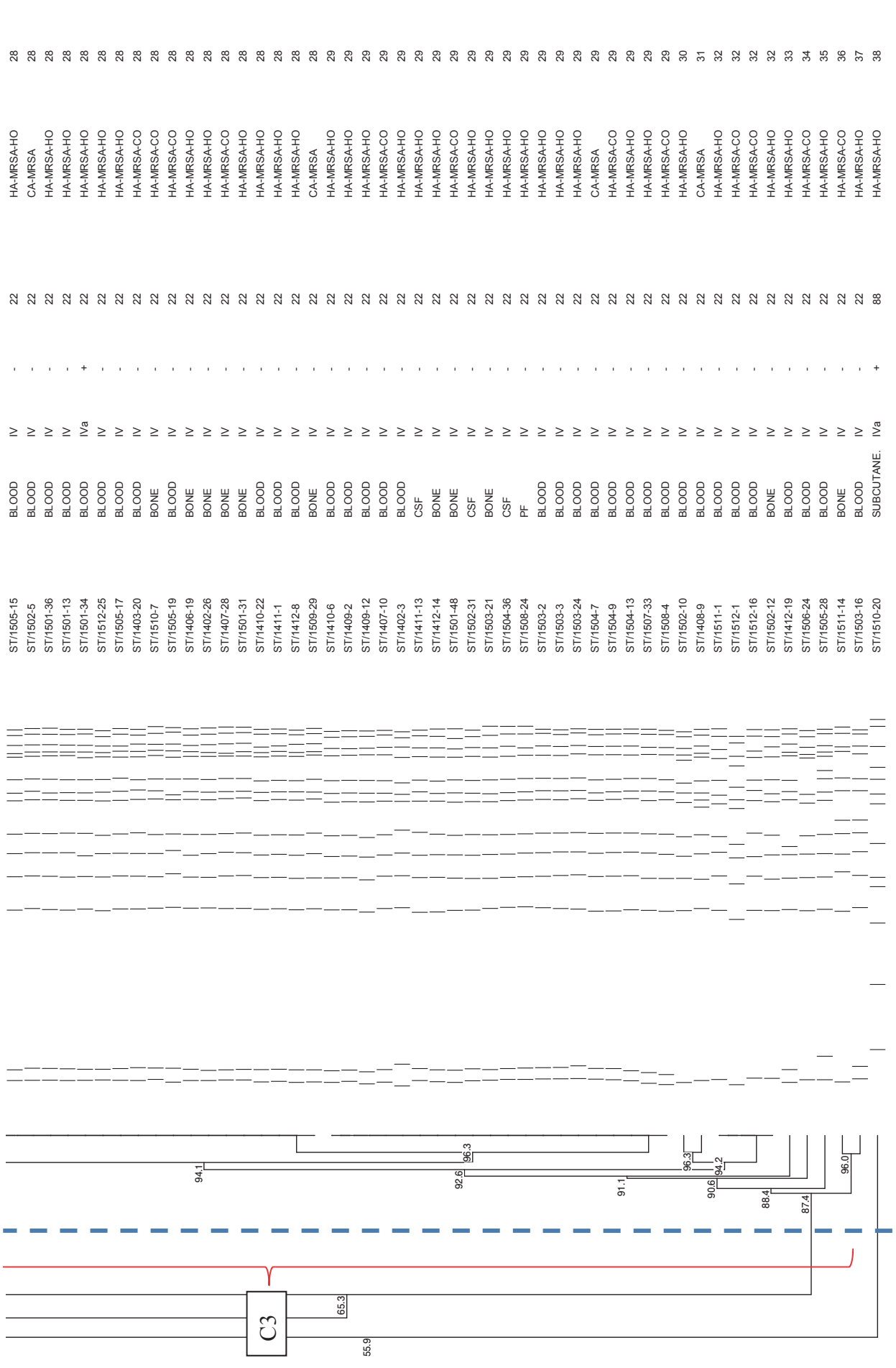


Fig. 1. Dendrogram of MRSA PFGE patterns generated by UPGMA clustering method using Dice coefficient. The dotted line indicates an arbitrary 80% similarity demarcation. C, cluster; SCCmec, staphylococcal cassette chromosome mec; PVL, panton-valentine leukocidin; ST, sequence type; MRSA, methicillin-resistant *Staphylococcus aureus*.

Table 3. Resistance patterns of MRSA isolates in 2014 and 2015

Antimicrobial agents	2014, *n* = 46 (%)	2015, *n* = 53 (%)	Total *N* (%)	*P*-value
SAM	35 (81.4)	47 (83.9)	82 (82.8)	0.740
ERY	35 (81.4)	50 (89.3)	85 (85.9)	0.264
GEN	17 (39.5)	11 (19.6)	28 (28.3)	**0.029**[1)]
FOX	43 (100)	56 (100)	99 (100)	
RIF	1 (2.3)	3 (5.4)	4 (4.0)	0.631
FUS	8 (18.6)	15 (26.8)	23 (23.2)	0.339
CIP	37 (86.0)	49 (87.5)	86 (86.9)	0.832
CLI	34 (79.1)	50 (89.3)	84 (84.8)	0.160
TZP	34 (79.1)	45 (80.4)	79 (79.8)	0.874
PEN	43 (100)	56 (100)	99 (100)	

Values are presented as *n* (%). MRSA, methicillin-resistant *Staphylococcus aureus*; SAM, ampicillin-sulbactam; ERY, erythromycin; GEN, gentamicin; FOX, cefoxitin; RIF, rifampicin; FUS, fusidic acid; CIP, ciprofloxacin; CLI, clindamycin; TZP, piperacillin-tazobactam; PEN, penicillin.
[1)]: Bold text indicates *P*-value < 0.05; which was considered to be statistically significant.

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 leucocidin (*pvl*) gene was detected in 6 (6.1%) isolates. It was distributed among 4 SCC*mec* type IVa isolates, 1 SCC*mec* type IVc isolate, and 1 SCC*mec* type V isolate. The prevalence of genes encoding virulence determinants in 2014 and 2015 is shown in Table 2. A statistical significant result was reported for the *icaA* gene ($p = 0.020$) from both years.

MLST: MLST analysis showed 13 STs among 99 MRSA clinical isolates (Table 1). The most prevalent MLST sequence type was ST22 (*n* = 55, 55.6%), followed by ST239 (*n* = 26, 26.3%). One novel sequence type, ST3547, was identified from a blood isolate collected in year 2015. The other 10 sequence types were ST6 (*n* = 4, 4.0%), ST188 (*n* = 2, 2.0%), ST45 (*n* = 2, 2.0%), ST5 (*n* = 2, 2.0%), ST8 (*n* = 2, 2.0%), ST1 (*n* = 1, 1.0%), ST772 (*n* = 1, 1.0%), ST1178 (*n* = 1, 1.0%), ST88 (*n* = 1, 1.0%), and ST769 (*n* = 1, 1.0%). MRSA isolates were clustered into 9 CCs based on the similarity at 6 of 7 loci. The major CC identified in this study was CC1 (ST1, ST769, and ST772). ST8 and ST239 were grouped into CC8, whereas ST5 and ST1178 grouped into CC5. ST22 was grouped into CC22, ST88 into CC88, ST45 into CC45, ST188 into CC188, and ST6 into CC6. The newly identified clone, ST3547 belonged to CC1047.

PFGE: The 99 MRSA isolates were grouped into 38 distinct pulsotypes comprised of 10-15 restriction fragments (Fig. 1). The pulsotypes were arbitrarily designated from 1 to 38, each defining a clone in accordance with the previously reported criteria. Based on 80% similarity, 3 major clusters were identified among the 99 clinical isolates (Clusters 1-3). The majority of the isolates (55.5%) were clustered in Cluster 3, followed by Cluster 1, which contained 21 isolates (21.2%), and Cluster 2, which contained 6 isolates (6.1%). The predominant pulsotypes were 28 (*n* = 20, 20.2%) and 29 (*n* = 20, 20.2%), both exhibiting ST22 profiles. Seventy-one isolates shared similar pulsotypes even though they were isolated from different sources with different modes of acquisition. Some isolates (pulsotypes 11, 12, 13, 22, 26, 27, 28, 29, and 32) from months apart shared similar pulsotypes. For instance, ST/1512-25 and ST/1505-17, isolated from the Nephrology, Endocrine, and Rheumatology (8TD) ward, belonged to pulsotype 28, even though they were 7 months apart. In addition, similar pulsotypes and modes of MRSA acquisition were observed in pulsotype 11, 28, and 29, which were cultured at about the same period of time. ST/1508-1 and ST/1509-1 demonstrated this phenomenon, where both isolates were isolated from the General Medical, Respiratory, and Gastroenterology (12U) ward and shared similar pulsotypes, SCC*mec* types, and sequence types.

Antibiotics susceptibility data: 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, resistances towards ampicillin-sulbactam (82.8%), erythromycin (85.9%), ciplofloxacin (86.9%), clindamycin (84.8%), and pipercacillin-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 SCC*mec* types have been identified worldwide, however, only 5 were reported in Malaysia. In this study, all 99 strains were successfully typed and 3 SCC*mec* types: III, IV, and V were detected. SCC*mec* type IV was the most predominant SCC*mec* type and was detected in 70.7% (*n* = 70) of the clinical isolates. These SCC*mec* type IV isolates showed diverse STs: 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 SCC*mec* type IV was HA-

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 SCC*mec* type III isolates were ST239. In contrast, SCC*mec* type V isolates were comprised of ST1, ST772, and ST3547. In the process of subtyping, 55 SCC*mec* type IV isolates were untypable by SCC*mec* IV subtyping; they might belong to novel SCC*mec* 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 SCC*mec* 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 SCC*mec* 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 SCC*mec* 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, SCC*mec*, 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 *sej* in the healthcare environment. On the other hand, different types of enterotoxin genes were harbored by strains with similar pulsotypes within the same cluster.

The majority of MRSA isolates in this 2-year study period belonged to the pandemic clone SCC*mec* IV-ST22 and most of them had pulsotype 28 and 29. Combined analyses based on PFGE, MLST, and SCC*mec* 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.

This is the first report on the emergence of ST8-MRSA-IVc (CA-MRSA), ST88-MRSA-IVa (HA-MRSA-HO), and ST769-MRSA-IVa (HA-MRSA-HO) isolates in Malaysia. A new sequence type, ST3547, was identified from a blood isolate collected from a patient undergoing hemodialysis on 2015. Rapid movement and ease of travel may be a contributing factor for the spread and dissemination of this clone in Malaysia.

Low resistance rates were reported for rifampicin, fusidic acid, and gentamicin in this center. However, high resistance rates were reported for ampicillin-sulbactam, erythromycin, ciprofloxacin, clindamycin, and pipercacillin-tazobactam. These findings are similar to those of the previously reported studies (23,30,31). Common prescription of these drugs against a variety of bacterial infections might also be a contributing factor. In assessing vancomycin susceptibility, all MRSA isolates remained susceptible. This finding was consistent with the findings reported by Sit et al. and Lim et al., who also studied MRSA isolates from the same center (21,23).

In conclusion, since the limited resources were available for the present study, we could not continue WGS to provide more discriminatory information. Despite of all limitations listed, the results affirm that community-associated variants are emerging and circulating in the hospital. Hence, continuous surveillance efforts are pivotal to monitor outbreaks and sporadic infections occurred in healthcare setting.

Acknowledgments The authors would like to thank University Malaya for facilities, utilities and Graduate Research Assistant Scheme (GRAS). This work was supported by FRGS, MOE (FP016-2014B) and UM BKP Grant (BK075-2016).

Conflict of interest None to declare.

REFERENCES

1. Song J-H, Hsueh P-R, Chung DR, et al. Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study. JAntimicrob Chemother. 2011;66:1061-9.
2. Chen CJ, Huang YC. New epidemiology of *Staphylococcus aureus* infection in Asia. Clin Microbiol Infect. 2014;20:605-23.
3. Stefani S, Chung DR, Lindsay JA, et al. Review: meticillin-

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 Ghani MK, et al. Characteristics of community- and hospital-acquired meticillin-resistant *Staphylococcus aureus* strains carrying SCC*mec* 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. 2004:9786-91.
7. International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC). Classification of Staphylococcal cassette chromosome*mec*(SCC*mec*): guidelines for reporting novel SCC*mec* 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 methicillin-susceptible clones of *Staphylococcus aureus*. J Clin Microbiol. 2000;38:1008-15.
9. David MZ, Daum RS. Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev. 2010;23:616-87.
10. Lim KT, Hanifah YA, Yusof MY, et al. 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. Diagn Microbiol Infect Dis. 2012;74:106-12.
11. Ghaznavi-Rad E, Nor Shamsudin M, Sekawi Z, et al. Predominance and emergence of clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* in Malaysia. J Clin Microbiol. 2010;48:867-72.
12. Milheirico C, Oliveira DC, de Lencastre H. Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* type IV in methicillin-resistant *Staphylococcus aureus*: 'SCC*mec* IV multiplex'. J Antimicrob Chemother. 2007;60:42-8.
13. Hisata K, Kuwahara-Arai K, Yamanoto M, et al. Dissemination of methicillin-resistant staphylococci among healthy Japanese children. J Clin Microbiol. 2005;43:3364-72.
14. Lina G, Boutite F, Tristan A, et al. Bacterial competition for human nasal cavity colonization: role of Staphylococcal *agr* alleles. Appl Environ Microbiol. 2003;69:18-23.
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, Godail-Gamot 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, ETD, and EDIN-B. Infect Immun. 2002;70:5835-45.
19. Kumar JD, Negi YK, Gaur A, et al. Detection of virulence genes in *Staphylococcus aureus* isolated from paper currency. Int J Infect Dis. 2009;13:e450-5.
20. Vasudevan P, Nair MK, Annamalai T, et al. Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol. 2003;92:179-85.
21. Sit PS, Teh CS, Idris N, et al. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. BMC Infect Dis. 2017;17:274.
22. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; 27th information supplement. Document M100. Wayne, PA: CLSI; 2017.
23. Lim KT, Hanifah YA, Mohd Yusof MY, et al. Comparison of methicillin-resistant *Staphylococcus aureus* strains isolated in 2003 and 2008 with an emergence of multidrug resistant ST22: SCC*mec* IV clone in a tertiary hospital, Malaysia. J Microbiol Immunol Infect. 2013;46:224-33.
24. Sun DD, Ma XX, Hu J, et al. Epidemiological and molecular characterization of community and hospital acquired *Staphylococcus aureus* strains prevailing in Shenyang, Northeastern China. Braz J Infect Dis. 2013;17:682-90.
25. Valle DL Jr, Paclibare PA, Cabrera EC, et al. Molecular and phenotypic characterization of methicillin-resistant *Staphylococcus aureus* isolates from a tertiary hospital in the Philippines. Trop Med Health. 2016;44:3.
26. Lim KT, Hanifah YA, Mohd Yusof MY, et al. Investigation of toxin genes among methicillin-resistant *Staphylococcus aureus* strains isolated from a tertiary hospital in Malaysia. Tropical biomedicine. 2012;29:212-9.
27. Hu DL, Omoe K, Inoue F, et al. Comparative prevalence of superantigenic toxin genes in meticillin-resistant and meticillin-susceptible *Staphylococcus aureus* isolates. J Med Microbiol. 2008;57:1106-12.
28. Sauer P, Sila J, Stosova T, et al. Prevalence of genes encoding extracellular virulence factors among meticillin-resistant *Staphylococcus aureus* isolates from the University Hospital, Olomouc, Czech Republic. J Med Microbiol. 2008;57:403-10.
29. 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.
30. Sit PS, Teh CSJ, Idris N, et al. Methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia: correlations between clinical, phenotypic, genotypic characteristics and mortality in a tertiary teaching hospital in Malaysia. Infect Genet Evol. 2018;59:132-41.
31. Institute for Medical Research, Ministry of Health Malaysia. National Antibiotic Resistance Surveillnace Report 2017. Available at < https://www.imr.gov.my/images/uploads/NSAR/NSAR_2017/NSAR_report_2017-edited-31.1.2019.pdf>. Accessed April 24, 2019.