Differences in antibiotic resistance profiles of methicillin-susceptible and–resistant Staphylococcus aureus isolated from the teaching hospital in Kuala Lumpur, Malaysia

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1. INTRODUCTION
Staphylococcus aureus is a common commensal found on the skin and human nasal cavity, with approximately 20–40% of the human population are S. aureus carriers that usually cause skin and soft-tissue infection [1]. Systemic infection of S. aureus leads to bacteremia, toxic shock syndrome, osteomyelitis, and endocarditis [2]. S. aureus secretes multiple toxins to evade the robust host’s immune system [3], and its ability to form biofilms on inserted devices such as catheter contributes to invasive infection [4]. The World Health Organization (WHO) has declared methicillin-resistant S. aureus (MRSA) a high priority in research and discovering novel antibiotics [5]. The prevalence of MRSA is the highest in the continents of Asia and America, although a decline has been observed throughout the years in some countries such as the USA and Taiwan [1]. MRSA infection contributes to approximately 20 000 deaths in the USA alone in 2017 [6]. Nationwide study in Malaysia revealed that MRSA infection prevalence has increased from 18% in 2016 to 19.8% in 2017, with most isolates were obtained from medical settings [7]. MRSA resistance to β-lactam antibiotics can be attributed to staphylococcal cassette chromosome mec (SCCmec), a mobile genetic element carries mec gene complex, ccr complex, and J regions that can be transferred horizontally from MRSA to methicillin-susceptible S. aureus (MSSA) [8]. Transfer of SCCmec from MRSA to MSSA poses a severe threat to the health-care setting, including an MRSA outbreak in health-care settings [9]. MRSA harboring mecA encodes penicillin-binding protein 2a (PBP2a) with a low affinity toward penicillin and other β-lactam antibiotics that lead to inactivation of this class of antibiotics [10]. To date, researchers have characterized thirteen SCCmec types to serve as predictive virulence markers for S. aureus [11].
Further, the rise of community-associated MRSA (CA-MRSA) has complicated MRSA transmission prevention as people with no known records of hospitalization are also predisposed to MRSA infection [12]. CA-MRSA is different from hospital-acquired MRSA (HA-MRSA) as it usually causes skin and soft tissue infection, while HA-MRSA infection is usually invasive and results in prolonged hospitalization [13]. MRSA isolated from Malaysian health-care settings usually harbors SCCmec type III, while isolates acquired from the community usually harbor SCCmec type IV or V [14]. The latest national report on MSSA and MRSA's resistance rates against antimicrobial agents in Malaysian health-care settings showed marked differences between them. MRSA exhibited higher resistance toward essential antibiotics for S. aureus treatments (erythromycin and gentamicin) compared to MSSA [7]. A recent study conducted in the Malaysian health-care setting showed the emergence of multidrug resistance MSSA [15]. The presence of resistance genes mediated by chromosome, transposon, or plasmid confers antibiotic resistance in S. aureus. Resistance to essential antibiotics, namely, erythromycin, tetracycline, and gentamicin, can be attributed to multiple variants of resistance genes, including erm, tet, and aminoglycoside modifying enzyme (Aac6′-D), respectively [16]. Although many studies have been conducted for MRSA in Malaysia, research on MSSA still lacks differences in antibiotic profiling and their resistance mechanisms. There is still a lack of studies to compare genotypes and phenotypes of antibiotic resistance in MSSA and MRSA. As the resistance mechanism in S. aureus is continuously evolving [1], understanding its mechanism is pertinent. Hence, this study aimed to compare current antibiotic resistance patterns in MSSA and MRSA and type SCCmec of MRSA isolates from our teaching hospital.

2. METHODOLOGY

2.1. Sample Collection of S. aureus

From July to October 2018, 50 MRSA and 50 MSSA isolates were obtained from Bacteriology Unit, Department of Diagnostic Laboratory Service, Hospital Canselor Tuanku Muhriz, Kuala Lumpur. All isolates were recultured onto Mueller-Hinton Agar (MHA) (Oxoid, Basingstoke, Hants, UK) for antibiotic susceptibility testing. MRSA isolates were identified using a cefoxitin disc (FOX) screen test.

2.2. Antimicrobial Susceptibility Testing

All S. aureus isolates were tested for antimicrobial susceptibility by disk-diffusion method on MHA agar plates following Clinical and Laboratory Standard Institute guidelines [17]. The antibiotics tested: Ciprofloxacin (5 µg), erythromycin (15 µg), fusidic acid (10 µg), gentamicin (10 µg), cefoxitin (30 µg), penicillin (10 U), clindamycin (2 µg), mupirocin (5 µg), teicoplanin (30 µg), rifampicin (5 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (1.25 µg), and linezolid (30 µg).

2.3. Genomic DNA Extraction of Bacteria

DNA extraction was conducted using a boiling method [18]. Briefly, several overnight culture colonies on Mannitol salt agar (MSA) plates were suspended into 100 µL distilled water, boiled in a water bath at 95°C for 10 min, and immediately placed in ice for 5 min. Next, the suspension was centrifuged at x10,000 g for 3 min. The supernatant was transferred into a fresh microcentrifuge tube. DNA concentration and purity were assessed by using a NanoDrop™ 2000 spectrophotometer (Life Technologies, Singapore), followed by storage at −20°C until use.

2.4. Identification of S. aureus isolates through Culture and 16S rRNA Sequencing

S. aureus isolates were identified by using coagulase, catalase tests, MSA agar, and 16S rRNA sequencing. These isolates were further inoculated onto MSA (Oxoid) and incubated at 37°C overnight. To verify S. aureus isolates, nine isolates from 100 isolates collected in this study were randomly selected and subjected to 16S rRNA gene amplification through polymerase chain reaction (PCR) using universal primers of 27F (5’-AGAGTTTGATCTGCTGAG-3’) and 1492R (5’-TACGTTACCTGTAGCCTT-3’). The final concentrations of primers and DNA were 0.4 µM (2 µL) and 50 ng (5 µL), respectively. Primers and DNA were added to ExTen mastermix (1st Base Asia, Singapore Science Park II, Singapore) for a final volume of 50 µL. PCR conditions were 94°C (initial denaturation) for 2 min, followed by 35 cycles of 94°C (1 min), 45°C (1 min), and 72°C (1 min), and final elongation at 72°C for 10 min. All PCR products were run on 1.0% agarose gel to view the amplified DNA band and then sent for sequencing to 1st Base Asia. Species identification was made by comparing 16S rRNA sequences with sequences from the Basic Local Alignment Search Tool database (National Centre of Biotechnology Information). All isolates were confirmed as S. aureus through molecular detection (sequence similarity more than 99%).

2.5. Screening of mecA gene in MRSA

Amplification of mecA gene was conducted via PCR. Primers used for amplification were MecA1 (5’-GTA GAA ATG ATG ACC GAA CGT CCG ATA A-3’) and MecA2 (5’-CCA ATT CCA CAT TGT TTT GGT CTA A-3’) [19]. The PCR reaction was performed in 25 µL reaction consisted of 2X ExTen mastermix (1st Base), 0.5 µM primers, 50 ng DNA template, and distilled water. PCR conditions were (1) initial denaturation (94°C for 4 min), (2) 30 cycles of denaturation (94°C for 45 s), (3) annealing (50°C for 45 s), and (4) extension (72°C for 60 s). The final extension was set at 72°C for 2 min. PCR products were electrophoresed on 1.0% agarose gel (Vivantis Technologies, Subang Jaya, Malaysia) and viewed under UV light for DNA band detection (Aplegen, California, USA). All MRSA isolates (100%; 50/50) harbored mecA in this study.

2.6. Multiplex PCR of ermA, ermC, tetK, tetM, and aacA-D Genes

S. aureus isolates that showed phenotypic resistance toward erythromycin, tetracycline, and gentamicin were selected for molecular analysis using multiplex and singleplex PCR as described previously [20]. PCR mixture consisted of 12.5µL ExTen 2X Master Mix (1st Base Asia), 0.5 µM primers (1.25 µL), 7.5 µL DNase-free water, and 50 ng DNA template (2.5 µL). The amplification process was performed using Bio-Rad T100™ thermal cycler (Bio-Rad, California, USA). PCR conditions for ermA, ermC, tetK, and tetM, and aacA-D detection were 5 min of initial denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 70 s, and extension at 72°C for 60 s. The final extension was set at 72°C for 10 min. For detection of tetM, PCR conditions were 6 min of initial denaturation at 94°C, followed by 34 cycles of denaturation at 95°C for 50 s, annealing at 55°C for 70 s, and extension at 72°C for 60 s. The final extension was set at 72°C for 8 min. The PCR products were analyzed on 1.5% (w/v) agarose gel (Vivantis Technologies). The gel was visualized under UV light of Omega Fluor Gel Documentation System (Aplegen).
2.7. SCCmec Typing Structure using Multiplex PCR

All MRSA isolates were subjected to SCCmec typing using multiplex PCR as previously described [21-26]. Briefly, nine set of primers were used to amplify SCCmec types I to V and the PCR reaction was conducted in a 25 μL reaction that consisted of 2X ExTEN PCR mastermix (1st Base Asia), nuclease-free water (Promega, Wisconsin, USA), 50 ng DNA template, and 0.5μM of primers. PCR conditions: (1) Initial denaturation at 95°C (15 min), (2) denaturation at 95°C for 30 s (30 cycles), and (3) annealing at 57°C for 90 s, and extension at 72°C for 90 s. The final extension was set at 72°C for 10 min. SCCmec typing was based on DNA band sizes, and isolates that gave no DNA bands were further examined by using method previously described by Ito et al. [27]. Briefly, eight primers were used to target ccr gene complex and four primers were used to target mec gene complex. The PCR reactions for amplification of ccr gene complex and mec gene complex were performed in 25 μL reaction consisted of 2X ExTEN mastermix (1st Base Asia), 50 ng of DNA, nuclease-free water, and 0.5 μM primers. PCR conditions for amplification of ccr gene complex were initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min. The final extension was set at 72°C for 2 min. For amplification of mec gene complex, the PCR conditions were initial denaturation at 95°C for 1 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min. The final extension was 72°C for 2 min. The PCR products were analyzed and visualized using 1.5% (w/v) agarose gel (Vivantis Technologies) and Omega Fluor Gel Documentation System (Aplegen), respectively.

2.8. Statistical Analysis

Comparison of antibiotic resistance profiling in MSSA and MRSA was analyzed using a two-tailed Fisher's exact test. P < 0.05 was considered significant. All analyses were conducted using Statistical Package for the Social Sciences (SPSS) software version 14.

3. RESULTS

3.1. Antimicrobial Susceptibility Test

MSSA isolates demonstrated the highest resistance to penicillin (84%; n = 42), followed by fusidic acid (24%; n = 12), tetracycline and ciprofloxacin with similar percentage (14%; n = 7), erythromycin (10%; n = 5), clindamycin (4%; n = 2), and gentamicin and rifampicin with similar percentage (2%; n = 1), respectively. No MSSA isolate was resistant to cefoxitin, mupirocin, teicoplanin, and co-trimoxazole [Table 1]. MRSA isolates demonstrated resistance to nine antibiotics which included penicillin (100%; n = 50), cefoxitin/methicillin (100%; n = 50), erythromycin (60%; n = 30), ciprofloxacin (56%; n = 28), clindamycin (30%; n = 15), and fusidic acid (10%; n = 5). A similar percentage of resistant isolates (8%; n = 4) were observed for clindamycin, rifampicin, and mupirocin, respectively. Similar to MSSA isolates, no MRSA isolates demonstrated resistance to teicoplanin and co-trimoxazole. Interestingly, all MRSA isolates were susceptible to tetracycline. Comparison of antibiotic resistance profiles between MSSA and MRSA isolates revealed that MRSA had significantly higher resistance to erythromycin (P < 0.0001), clindamycin (P = 0.0009), and ciprofloxacin (P < 0.0001) than MSSA. Meanwhile, MSSA had a significantly higher percentage of resistance to tetracycline than MRSA isolates (P = 0.0125). S. aureus isolates with resistance to at least one antimicrobial agent in at least three antimicrobial classes were previously defined as multidrug-resistant [28]. By following this guideline, 14% (7/50) of MSSA isolates were multidrug-resistant. Table 2 shows five MSSA isolates (MS26, MS32, MS44, MS47, and MS49) were resistant to three antimicrobial categories, while two strains were resistant to four antimicrobial categories (MS30 and MS48). Meanwhile, 66% (33/50) of MRSA were multidrug-resistant. Of which, three (MR8, MR44, and MR46) were resistant to four antimicrobial categories.

3.2. Detection of ermA, ermC, tetK, tetM, and aacA-D in MSSA and MRSA Isolates

MSSA and MRSA isolates that showed resistance to erythromycin, gentamicin, and tetracycline, respectively, at the phenotypic level, were selected to detect ermA, ermC, tetK, tetM, and aacA-D genes. We observed the presence of ermC (299 base pair) in all five MSSA isolates that showed resistance to erythromycin at the phenotypic level. However, none of them harbored ermA gene. A similar result was observed in MRSA isolates, in which 27 out of 31 isolates harbored ermC while no isolate harbored ermA [Table 3]. Among seven MSSA isolates resistant to tetracycline at phenotypic level, only one isolate harbored tetK (190 base pair). As expected, no tetracycline resistance gene was detected in MRSA isolates. Resistance gene to aminoglycoside (aacA-D) was not detected in all MSSA, and MRSA examined.

3.3. SCCmec Typing in MRSA Isolates

SCCmec typing in MRSA isolates revealed 48 strains harbored SCCmec type IV and two strains harbored SCCmec type II [Figure 1]. Interestingly, MRSA strains with SCCmec type II (MR34 and MR45) were multidrug-resistant to four antimicrobial classes. SCCmec types I, III, and V were not detected among MRSA examined.

4. DISCUSSION

Data on the comparison of antibiotic resistance profiling among MSSA and MRSA isolates from health-care centers in Malaysia are lacking. Hence, we conducted this study to profile antibiotic resistance susceptibility of MSSA and MRSA and type SCCmec elements in MRSA isolates. No MRSA and MSSA isolates were resistant to vancomycin in this study, consistent with the previous studies in Malaysia [29-31]. Resistance to penicillin was the highest
in both MSSA and MRSA groups, consistent with findings published previously from a large scale study of antibiotic resistance profiling among S. aureus isolates in Malaysia [7]. All MRSA strains were resistant to penicillin, indicating that they all express PBP2a protein that confers resistance to β-lactam antibiotics. Similar to previously published data, high resistance of MRSA strains to erythromycin was observed [32]. Consistent with the previous report, a low resistance rate to gentamicin and fusidic acid was observed among MSSA and MRSA isolates [7]. The resistance of S. aureus to gentamicin has been observed to decline throughout the years in Malaysian health-care settings, suggesting the efficacy of antimicrobial stewardship in Malaysia [14]. We found that all MRSA strains were sensitive to tetracycline. Data on the prevalence of tetracycline resistance among

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MRSA isolates in Malaysia are lacking. The latest national report on antimicrobial resistance of S. aureus did not include findings on tetracycline [7]. However, a recent study conducted in East Coast Malaysia showed that tetracycline resistance among MRSA isolates was low at 7.6% [15]. This finding suggests the use of tetracycline as treatment of MRSA in our health-care setting. More studies on the susceptibility of MRSA strains to tetracycline should also be conducted in the future. Consistent with the previous findings [15], a high percentage of MRSA (66%) with multidrug resistance was observed in this study. Interestingly, 14% of MSSA isolates were multidrug-resistant, suggesting further surveillance of MSSA isolates in our health-care setting.

Table 2: Multidrug resistance analysis of MSSA (n=50) and MRSA (n=50) isolates as determined by antimicrobial susceptibility test.

| Antibiotics resistance | Total resistance isolates (n) |
|-------------------------|------------------------------|
| No drug resistance      | 3                            |
| PEN                     | 23                           |
| TETRA                  | 1                            |
| FA                     | 1                            |
| ERY                    | 1                            |
| PEN, TETRA             | 3                            |
| PEN, FA                | 7                            |
| PEN, CIP               | 2                            |
| ERY, FA                | 1                            |
| FA, CIP                | 1                            |
| PEN, GEN, RIF          | 1                            |
| PEN, TETRA, FA        | 1                            |
| PEN, ERY, CLIN       | 1                            |
| PEN, TETRA, CIP    | 2                            |
| PEN, ERY, CLIN, CIP | 1                            |
| PEN, ERY, FA, CIP | 1                            |
| MRSA (N=50)            |                              |
| PEN, FOX                | 1                            |
| PEN, FOX, CIP         | 9                            |
| PEN, FOX, ERY         | 6                            |
| PEN, FOX, FA          | 1                            |
| PEN, FOX, GEN, CIP   | 1                            |
| PEN, FOX, RIF, CIP  | 4                            |
| PEN, FOX, ERY, MUP  | 4                            |
| PEN, FOX, ERY, CLIN | 6                            |
| PEN, FOX, ERY, CIP  | 6                            |
| PEN, FOX, GEN, FA | 1                            |
| PEN, FOX, CLIN, FA | 1                            |
| PEN, FOX, ERY, CLIN, CIP | 7                         |
| PEN, FOX, GEN, FA, CIP | 1                         |
| PEN, FOX, ERY, CLIN, FA | 1                        |
| PEN, FOX, ERY, CLIN, GEN, FA | 1                      |

Table 3: Comparison between antibiotic resistance at phenotypic and genotypic levels in MSSA and MRSA strains.

| Resistance genes | No of isolates with detected genes (n per total) |
|------------------|-----------------------------------------------|
| ermA             | 0/5                                           |
| ermC             | 5/5                                           |
| tetK             | 1/7                                           |
| tetM             | 0/7                                           |
| aacA-D           | 0/1                                           |
| ermA             | 0/31                                          |
| ermC             | 27/31                                         |

MS denotes the given strain name to methicillin-susceptible S. aureus and MR denotes given strain name to methicillin-resistant S. aureus. MRSA: Methicillin-resistant Staphylococcus aureus, MSSA: Methicillin-susceptible Staphylococcus aureus.
The majority of MRSA harbored SCCmec type IV in our clinical setting. A previous study on SCCmec typing in MRSA collected from our healthcare setting revealed a high prevalence of MRSA harbored SCCmec type III [29]. This finding suggests that MRSA with SCCmec type IV is replacing MRSA with SCCmec type III in our setting. SCCmec types I to III are mostly associated with HA-MRSA while types IV and V are mostly associated with CA-MRSA in our country [14]. Our findings indicate that MRSA with SCCmec type IV isolated from this study might belong to the CA-MRSA type. Further study to characterize clonal type, virulence factors, and SCCmec elements of MRSA isolates in our setting should be conducted.

This study employed the detection of resistance genes in erythromycin, tetracycline, and gentamicin as National Antibiotic Guideline recommends these antibiotics for the treatment of MRSA infection [33]. Antibiotic resistance gene toward erythromycin (ermC) was detected in all MSSA strains that showed resistance to erythromycin at the phenotypic level. The majority of MRSA strains from this study harbored ermC instead of ermA. This result is contrary to what was reported previously from another study that also investigated MRSA strains isolated from Kuala Lumpur, in which ermA was predominant [34]. However, there were MRSA isolates with a lack of ermA or ermC genes from our study. Lack of ermA or ermC in MRSA isolates with phenotypic resistance to erythromycin suggests that these isolates may harbor other variants of erm gene. We chose to amplify tetK and tetM variants of tetracycline-resistant S. aureus isolated from this study because a previous study conducted in Malaysia revealed all tetracycline-resistant S. aureus harbored either tetK or tetM [34]. Investigation on tetracycline resistance gene among MSSA isolates revealed that only one out of seven MSSA isolates that showed resistance to tetracycline at phenotypic level harbored tetK at a genotypic level while tetM was not detected. tetK is a plasmid-mediated resistance gene involved in efflux pump protein, while tetM resistance is transposon-mediated resistance gene involved in the protection of ribosomal translation [16]. Other variant genes apart from tetK or tetM might be present in S. aureus strains isolated from our hospital. Besides, other molecular mechanisms not involving tet gene presence may play a role in conferring tetracycline resistance in S. aureus isolated from our setting. Data on tetracycline resistance among S. aureus in Malaysia is limited. To the best of our knowledge, no report of tetracycline antibiotic resistance gene among MSSA isolates has been made in Malaysia.

Further studies to profile tetracycline resistance among S. aureus isolates in Malaysian healthcare settings should be conducted. This study also revealed no detection of AacA-D gene in gentamicin-resistant MRSA and MSSA. No study has reported on the prevalence of AacA-D gene in S. aureus isolates collected from Malaysia. Resistance to aminoglycosides can occur through expression of resistance genes mediated by a plasmid, transposon, or SCCmec elements [35]. AacA-D gene is mediated by transposon and usually confers resistance of S. aureus against gentamicin based on studies conducted previously in other regions [36,37]. Lack of AacA-D gene in gentamicin-resistant S. aureus isolates from our study suggests the presence of other resistance genes or resistance mechanisms in S. aureus isolates from our healthcare setting.

5. CONCLUSION

We have compared the current distribution of antibiotic resistance patterns among MSSA, and MRSA isolates from our teaching hospital, emphasizing the relatedness of phenotypic and genotypic antibiotic resistance profiles. The emergence of multidrug resistance in MSSA warrants further surveillance. This study also revealed that MRSA harboring SCCmec type IV is currently predominant in our teaching hospital, indicating the current dominance of CA-MRSA in our setting. Future in-depth studies on S. aureus, particularly MSSA isolates from our population using other molecular techniques are warranted.

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7. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

Not applicable.

10. PUBLISHER’S NOTE

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