Sains Malaysiana 51(3)(2022): 795-801
http://doi.org/10.17576/jsm-2022-5103-13

Heterogeneous Vancomycin-Intermediate *Staphylococcus aureus* (HVISA) at a Tertiary Hospital in Malaysia

(Heterogen Pertengahan Vankomisin *Staphylococcus aureus* (HVISA) di Sebuah Hospital Tertier di Malaysia)

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Received: 1 February 2021/Accepted: 26 July 2021

ABSTRACT

Methicillin-resistant *Staphylococcus aureus* remains a global problem. The emergence of reduced susceptibility to Vancomycin in MRSA strains, leads to treatment failure and prolonged hospital stay. Therefore, we aimed to determine the strains with reduced susceptibility among MRSA clinical isolates. *S. aureus* isolates were collected from identified clinical samples. Antibiotic susceptibility was tested using the Kirby-Bauer method. MRSA strains were confirmed using PCR for *mec*A gene and subjected to the Epsilometer test (Etest®*)* for determination of Vancomycin minimum inhibitory concentration (MIC). Isolates with intermediate or reduced susceptibility were subjected to broth microdilution (BMD) and further confirmed by population analysis-area under curve (PAP-AUC) testing. Disc diffusion tests showed that all isolates (n = 105; 100%) were susceptible to Rifampicin, followed by Trimethoprim/ Sulfamethoxazole. Meanwhile, a high resistance rate was demonstrated against Penicillin (n = 93; 88.6%). Among all isolates, only 26.0% (n = 27) were MRSA. According to the Vancomycin MIC value by Etest®*,* only two strains (A3, A106) had intermediate susceptibility, and one strain (A30) had reduced susceptibility to Vancomycin (MIC 3 µg/mL). No susceptibility to Vancomycin was identified among strains using the BMD method. According to the PAP-AUC method, it was confirmed that strain A3 was a heterogeneous VISA strain. Vancomycin Etest®, is a reliable screening test for VISA detection. Vancomycin BMD result was not in agreement with the Vancomycin Etest® result. PAP-AUC, the gold standard test used to detect Vancomycin resistance, should be conducted whenever possible for further confirmation and epidemiological record.

Keywords: Antibiotic resistance; hetero-VISA; MRSA; *mec*A; *Staphylococcus aureus*

ABSTRAK

Staphylococcus aureus rintang metisilin (MRSA) masih menjadi masalah klinikal global. Kejadian kerintangan terhadap strain Vankomisin MRSA menyebabkan kegagalan rawatan dan tempoh tinggal pesakit di hospital yang berpanjangan. Kajian ini dijalankan bagi mengenal pasti strain klinikal MRSA yang mempunyai penurunan kerentanan di sebuah hospital tertier di Malaysia. Strain *Staphylococcus aureus* diperoleh daripada pelbagai spesimen klinikal yang dikenal pasti secara fenotip. Ujian kerentanan terhadap antibiotik dijalankan dengan kaedah Kirby-Bauer. Strain (MRSA) disahkan oleh pengesanan gen *mec*A melalui tindak balas berantai polimerase (PCR), seterusnya penentuan kepekatan perencatan minimum Vankomisin (MIC) dijalankan melalui Etest®. Mikrodilusi kaldu (BMD) dijalankan bagi strain kerentanan pertengahan atau berkurangan. Selanjutnya, kerintangan disahkan oleh kawasan analisis populasi di

bawah ujian keluk (PAP-AUC). Ujian penyebaran cakera menunjukkan semua strain (n = 105, 100%) rentan terhadap Rifampicin dan diikuti oleh Trimethoprim/Sulfamethoxazole. Walau bagaimanapun, kerintangan tinggi terhadap Penicillin (n = 93, 88.6%) ditunjukkan. Strain MRSA merangkumi 26.0% (n = 27) jumlah dikaji. Nilai MIC Vankomisin oleh Etest® dua strain (A3, A106) mempunyai kerentanan pertengahan (VISA) dan satu strain (A30) mempunyai pengurangan kerentanan terhadap Vankomisin dengan MIC 3 µg/mL. Tetapi, BMD tidak menunjukkan pengurangan kerentanan terhadap Vankomisin. Kaedah PAP-AUC mengesahkan bahawa strain A3 adalah hetero- Vankomisin pertengahan *S. aureus* (hVISA). Vankomisin Etest® adalah ujian saringan yang boleh dipercayai untuk pengesanan Vankomisin pertengahan *S. aureus* (VISA). Hasil mikrodilusi kaldu Vankomisin tidak sama dengan hasil Vankomisin Etest®. PAP-AUC sebagai ujian piawai menguji kerintangan terhadap Vankomisin disarankan bagi pengesahan tepat serta pengenalpastian epidemiologi kerintangan Vankomisin.

Kata kunci: Hetero-VISA; MRSA; *mec*A; rintangan antibiotik; *Staphylococcus aureus*

Introduction

Staphylococcus aureus, as a major pathogen of humans, is an issue of global healthcare concern. As part of the normal human flora, it colonises its host asymptomatically. Occasionally, *S. aureus* causes various infections from the relatively less severe minor skin and soft tissue infections to the life-threatening scalded skin syndrome, bacteraemia, and pneumonia. Originally, for various *S. aureus* infections, Penicillin was the first-choice drug. However, the emergence of β-lactamase producing strains in 1942 made them Penicillin-resistant. Nowadays, over 95% of *S. aureus* from humans are proven to be Penicillin-resistant (Fuda et al. 2005; Tang et al. 2014). The drugs of choice for treating *S. aureus* infections were initially the β-lactam antibiotics, but a slightly different strain of this organism, the Methicillin-resistant *S. aureus* (MRSA), emerged in 1961 and proved resistant to all members of the class of β-lactam antibiotics. For more than half a century, MRSA has been a clinical problem worldwide. The set of genes within the operons *bla* and *mec* are the molecular basis for the incidentally inducible broad resistance of MRSA (Boudreau et al. 2015). In MRSA, the mediation of Methicillin resistance is brought about by the presence of β-lactam antibiotic-low affinity 78-kDa penicillin-binding protein PBP2 (or PBP2a). The gene *mec*A codes for PBP2a (Moreillon 2008). The *mec*A gene is located within a mobile genetic element (from 21kb to 67kb) known as Staphylococcal Cassette Chromosome *mec* elements (SCC*mec*) (Ammons et al. 2010).

In the National Surveillance on Antimicrobial Resistance Report 2018 (NSAR 2018), a total of 45,813 *S. aureus* strains were isolated from clinical samples. Majority of the isolates were from blood, swab/wound and pus (23.5%, 18.8%, and 18.0%, respectively). There was an increasing resistance trend observed with Penicillin G, whereas a decreasing trend in resistance was observed with Clindamycin, Erythromycin, Gentamicin, Fusidic acid and Linezolid. MRSA prevalence ranged from 17.2% to 28% in Malaysia. The national prevalence rate of MRSA was 19.4%, slightly lower than that of the previous year, which was 19.8%. The majority of cases included patients in the medical, surgical wards and the intensive care units.

Vancomycin, a glycopeptide, was first introduced to treat Gram-positive bacteria in 1958 and it remains the drug of choice for MRSA infections (NSAR 2018). In 1996, Mu50, the first clinical vancomycin-intermediate *S. aureus* (VISA) strain to be isolated, had a vancomycin minimum inhibitory concentration (MIC) of 8 mg/L and Mu3, the hetero-VISA (hVISA) strain had an MIC of 2 mg/L. The hVISA is the precursor of VISA, carrying subpopulations of VISA in its colonies. Due to their small numbers in hVISA, these VISA colonies could not be detected using conventional MIC determination methods in the diagnostic microbiology laboratory. Subsequently, to reflect the frequent therapeutic failure of Vancomycin against MRSA, the Clinical Laboratory Standards Institute (CLSI) has redefined Vancomycin breakpoints as susceptible at a Vancomycin broth MIC of ≤2 µg/mL, intermediate at a Vancomycin broth MIC of 4-8 µg/mL and resistant to Vancomycin broth MIC of ≥16 µg/mL (CLSI 2016; Song et al. 2004).

Several vancomycin-resistant *Staphylococcus aureus* (VRSA) strains have been reported in the USA, which include one community-associated strain (Walters et al. 2015). In 2018, a clinical VRSA strain with an MIC of ≥32 mg/L was isolated in Indonesia, our neighbouring country (Ramazoni et al. 2018). Conversely, in the same year, the first VISA strain was isolated in Malaysia (Hashim et al. 2018). Presently, no VRSA has been detected. With the occurrence of alarming incidences, surveillance is paramount to curb the spread of MRSA

and VRSA in the Malaysian setting. Therefore, we report the prevalence of MRSA and our experience of hVISA detection among *S. aureus* strains isolated from February 2016 to February 2017 in a tertiary hospital in Malaysia.

Materials and Methods

Study design and identification of isolates

A total of 105 *S. aureus* strains were collected from various clinical samples between February 2016 and February 2017 at a tertiary hospital in Malaysia (Hospital A). The specimens were cultured overnight on Mannitol Salt Agar (Oxoid, UK) in ambient air at 37 ºC. Isolates were identified based on colony morphology and colour change of medium (from red to yellow) due to mannitol fermentation. Further recognition was conducted through routine biochemical identification tests, such as Gram staining, catalase test and coagulase test. Pure colonies were duplicated and preserved in trypticase soy broth (Oxoid, UK) with 30% glycerol at -80 ºC (Arora & Arora 2007).

Disc diffusion test

S. aureus suspension was prepared from an overnight culture with turbidity identical to 0.5 McFarland standards (Hardy diagnostics, USA) and inoculated on Mueller–Hinton agar plates (Oxoid, UK). The antibiotic discs were placed on the dried inoculated agar surface and plates were incubated at 37 ºC for 18-24 h to study antibiotic susceptibility pattern against the following antibiotics: Cefoxitin [for detecting Oxacillin (Methicillin) resistance], Clindamycin, Erythromycin, Fusidic acid, Gentamicin, Penicillin, Rifampicin and Trimethoprim/Sulfamethoxazole. ATCC 25923 *Staphylococcus aureus* was used as a quality control strain. The result was interpreted according to the CLSI criteria (CLSI 2016).

Detection of *mec*A methicillin-resistance gene

The standard protocol was used for DNA extraction with minor modifications (CDC 2015). DNA purity and concentration were measured using NanoDrop (Thermo Scientific Technologies LLC, USA). Extracted DNA was stored at -20 ºC. All clinical *S. aureus* isolates resistant to Methicillin were subjected to polymerase chain reaction (PCR) assay for *mec*A gene detection. A pair of primers was used to amplify the *mec*A gene (Table 1). The following reaction mixture was added: 12.5 µL master mix (Thermo Scientific™), 0.3 µL (10 µM) of each primer, 1 µL (45-408 µg/mL) DNA template and 10.9 µL nuclease-free water was used to top up 25 µL. *S. aureus* ATCC 33591, and nuclease-free water was used as the positive and negative control. PCR Bio-Rad MyCycler™ Thermacycler (Bio-Rad, USA) was set at the following conditions: primary denaturation at 95 ºC for three minutes, followed by 40 cycles (95 ºC for 30 s, 50 ºC for 30 s and 72 ºC for one minute) and a final extension at 72 ºC for five minutes. Amplified DNA fragment with 147 bp size was reported as *mec*A gene positive (Mat Azis et al. 2014).

Epsilometer test (Etest®)

Vancomycin MIC was measured for methicillin-resistant strains. *S. aureus* ATCC 29213 served as a quality control strain. The visual breakpoint was determined by measuring an elliptical zone of inhibition. MIC was read where the growth intersected the Etest® strip (0.016-256 ug/mL; Liofilchem, Italy). Isolates with MICs ≤2, 4-8 and ≥16 ug/mL were considered as susceptible, intermediate and resistant to Vancomycin, respectively, according to the breakpoints published by CLSI, 2016 (CLSI 2016).

Confirmation of VISA/hVISA

Strains with intermediate or reduced susceptibility to Vancomycin (as determined by Etest®) were subjected to broth microdilution (BMD) to further verify the MIC value of the organism (Perovic et al. 2017). Population Analysis-Area Under the Curve (PAP-AUC) testing, which is still considered the gold standard phenotypic method to confirm further hVISA strains, was also performed. Mu50 and Mu3 were used as VISA and hVISA control strains, respectively (Wootton et al. 2001).

Results

Among the 105 clinical isolates of *S. aureus* (n = 27), 26.0% were found to be Methicillin resistant. PCR results showed that all MRSA isolates harboured the *mec*A gene. The highest MRSA prevalence rate was from wound and blood specimens (n = 13, n = 7, respectively) 48.1%, and 25.9%, respectively. Disc diffusion showed that all isolates (n = 105; 100%) were susceptible to Rifampicin followed by Trimethoprim/Sulfamethoxazole (n = 104; 99%). The highest resistance proportion among all isolates was recorded against Penicillin (n = 93) 88.6%. The results for all isolates against all antibiotics are represented in Table

2. Etest® result showed that only two MRSA (7.4%), one each from blood (A3) and wound (A106), had intermediate resistance to Vancomycin (MIC = 4 and 6 µg/mL, respectively), and another isolate (A30) (3.7%), from nasopharyngeal aspirate exhibited reduced susceptibility to Vancomycin with MIC 3 µg/mL. These three strains were found to be Vancomycin susceptible *S. aureus* using BMD (Table 3). Further confirmation by PAP-AUC showed that one VISA strain (A3) had a PAP-AUC ratio of 0.9 and was reported as a hVISA strain (Figure 1).

TABLE 1. Sequence of oligonucleotide primers use for detection of *mec*A gene

Primer	Sequence (5'-----3')	Size (bp)	Reference
*mec*A	F: AAA ACT AGG TGT TGG TGA AGA TAT ACC R: GAA AGG ATC TGT ACT GGG TTA ATC AG	147	(Mat Azis et al. 2014)

TABLE 2. Antibiotic susceptibility pattern of clinical *Staphylococcus aureus* isolates from a tertiary hospital in Malaysia

Antimicrobial agent (g)	Susceptible n (%)	Intermediate n (%)	Resistant n (%)
Cefoxitin (30)	78 (74%)	0 (0%)	27 (26.0%)
Clindamycin (2)	88 (83.8%)	1 (0.95%)	16 (15.2%)
Erythromycin (15)	76 (72.4%)	4 (3.8%)	25 (23.8%)
Fusidic acid (10)	88 (83.8%)	0 (0%)	17 (16.2%)
Gentamicin (10)	100 (95.2%)	0 (0%)	5 (4.8%)
Penicillin (10 units)	12(11.4%)	0 (0%)	93 (88.6%)
Rifampicin (5)	105 (100%)	0 (0%)	0 (0%)
Trimethoprim/ Sulfamethoxazole (1.25 / 23.75)	104 (99%)	1 (0.95%)	0 (0%)

TABLE 3. Vancomycin MIC value of MRSA isolates by Etest® and broth microdilution (BMD) test

Specimen type	Strain No.	Vancomycin MIC Etest® µg/mL	Interpretation	Vancomycin MIC BMD-test µg/mL	Interpretation
Blood	A3	4.0	VISA	2.0	VSSA
Nasopharyngeal Aspirate	A30	3.0	Reduce susceptibility to vancomycin	1.0	VSSA
Wound	A106	6.0	VISA	2.0	VSSA

(S: ≤ 2, I: 4-8, R: ≥ 16) CLSI 2016 breakpoint (CLSI 2016)

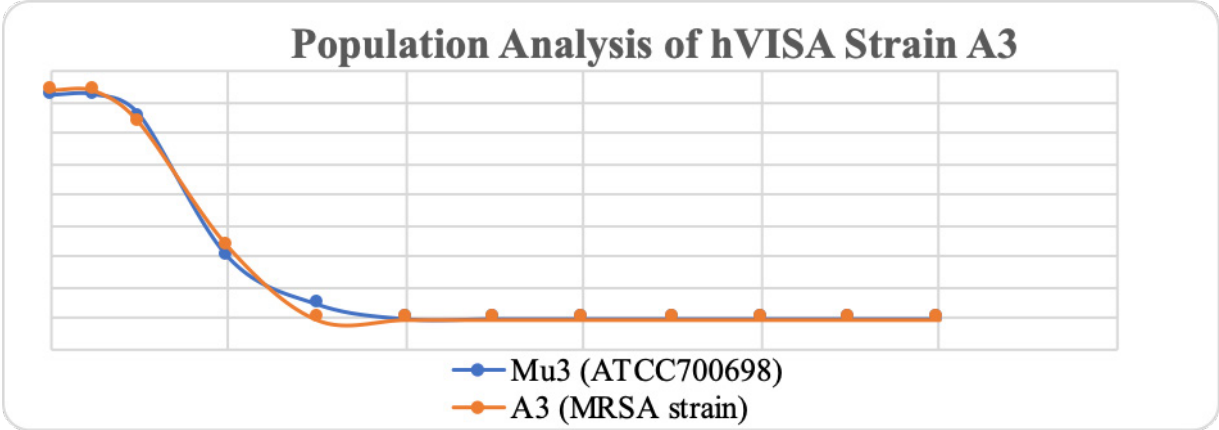

FIGURE 1. Demonstrates the population analysis area under curve of control strain Mu3-hVISA (ATCC700698) and tested strain A3 (MRSA strain) with PAP-AUC ratio 0.9 (hVISA)

DISCUSSION

Antimicrobial susceptibility profiles serve as a helpful guideline for selecting an appropriate antibiotic to treat *S. aureus* infection. Antibiotics tested in this study were chosen from CLSI standard guidelines and are routinely used in diagnostic laboratories of hospitals in Malaysia. The proportion of MRSA isolates were significantly higher in our study (n = 27), 26.0%, compared to the NSAR 2016 report, which was 18.0% (NSAR 2016). The resistance gene, *mec*A, was detected in all phenotypic MRSA strains (100%) in concordance with the 'Cefoxitin disc diffusion method'. This further confirms the reliability of the Cefoxitin disc diffusion method for detecting MRSA in the absence of the *mec*A gene detection method in the diagnostic laboratory. This corroborates with the study by Sabet et al. (2007) where the *mec*A gene was detected among all phenotypic MRSA isolates using triplex real-time PCR assay. Anand et al. (2009) reported similar findings, stating that all 32 Cefoxitin resistant strains identified using the disc diffusion method were *mec*A gene positive. Moreover, majority of *S. aureus* isolates were Penicillin-resistant, 88.6%, which was slightly lower compared to another study in Malaysia, conducted by Rohani et al. (2000). In a study in China, Yang et al. (2016) showed a slightly lower percentage of resistance to Penicillin 84.09%.

For a long time, Vancomycin has been considered to be a drug of choice for MRSA infection treatment and remains the mainstay for MRSA treatment. However, its efficacy has been questioned since the appearance of the strains with reduced susceptibility to Vancomycin, designated as Mu50 (VISA) and Mu3 (hVISA), was reported in Japan (Hiramatsu et al. 2014, 1997a, 1997b). The reduced susceptibility of these strains to Vancomycin is not associated with the *van*A gene, which is found to be the main cause of Vancomycin resistance in certain enterococci species and has been infrequently found in VRSA (Cui et al. 2006; Howden et al. 2008), but is a result of numerous phenotypic changes (Rong & Leonard 2010). Even though VISA and VRSA strains are still rare, reporting of actual antibiotic susceptibility of culture-confirmed MRSA strains is more relevant than information on the clone. Local susceptibility profiles are critical in selecting the most appropriate antibiotic for MRSA (Wilcox et al. 2019). Analysis suggests a correlation between the Vancomycin MIC and the clinical outcome (Hiramatsu et al. 2014; Wilcox et al. 2019). Accurate MIC measurement is essential to deliver the most appropriate treatment for MRSA, thus increasing the judicious use of Vancomycin and reducing the emergence of resistant strains.

Precise determination of Vancomycin MIC for *S. aureus* is associated with methodological difficulties. Antibiotic susceptibility test can be done via manual, e.g. disc diffusion, BMD and Etest®, or automated methods, e.g. Vitek®2, BD Phoenix™, Microscan and Sensititre™ (Adam et al. 2010). In 2014, Pulingam et al. reported three VISA strains from Hospital Pulau Pinang using Etest® for MIC determination and in 2018, Hashim et al. reported a VISA strain in Hospital Melaka. In our study, Etest® MIC identified two VISA strains and one reduced susceptibility strain. We proceeded with another manual method, i.e. BMD, which showed that the three strains were susceptible. Subsequently, PAP-AUC

technique confirmed that one VISA strain was a hVISA; hVISA was identified in Hospital Kuala Lumpur a decade ago. Nevertheless, there were no reports about hVISA being isolated recently in Malaysia. These observations indicate that there are discrepancies in the MIC values obtained by various methods.

While Etest® does not provide accurate MIC readings, it can be used as a screening test for VISA strain pending confirmation by BMD testing and is useful as a preliminary determinant of potential MRSA treatment failure in the clinical setting. PAP-AUC test can be conducted subsequently for hVISA strain confirmation and epidemiological purposes. This further reiterates the paramount importance of the recommendations by Wilcox et al. (2019) that if an anomalous MIC is found in the susceptible range (> 1 g/mL and ≤ 2 g/mL), a re-test by a different method should be performed to further determine the MIC values. The method should be manual, i.e. BMD or Etest®. Henceforth, potential therapeutic failure and limitation of Vancomycin as a treatment option should be forewarned to the treating clinician. Regular quality control tests for each method should be in place to ensure that values are genuine and reliable to guide antibiotic therapy.

ACKNOWLEDGEMENTS

Our utmost gratitude to the late Professor Dr. Keiichi Hiramatsu (Juntendo University, Tokyo, Japan), staff of Medical Microbiology Unit, Department of Pathology, Hospital Serdang, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, UPM and Raja Mohd Fadhil B Raja Abd Rahman, UKM Medical Molecular Biology Institute for their kind assistance and cooperation. This study was partially funded by Universiti Putra Malaysia Research Initiative Grant (GP-IPS /2016/ 9495400).

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