

Phenotypic and Genotypic Characterization, and Detection of PVL Encoding Gene in Methicillin Resistant *Staphylococcus Aureus* Strains Isolated From Patients Admitted to a Tertiary Hospital In Kuantan, Malaysia

Mohammad Hanif Bariman^a, Mohammed Imad A. Mustafa Mahmoud^b, Hairul Aini binti Hamzah^c

^aDepartment of Microbiology, Faculty of Medicine, Kabul Medical University, 24000 Kabul, Afghanistan.

^bDepartment of Basic Medical Science, Faculty of Medicine, IIUM, 25200 Kuantan, Pahang, Malaysia.

^cDepartment of Basic Medical Science, Faculty of Medicine, IIUM, 25200 Kuantan, Pahang, Malaysia

ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus aureus* is globally a major public health threat. Resistance to methicillin originates from a modified protein (PBP2a) encoded by the *mecA* gene. The *PVL* gene as an important virulence factor increases the pathogenicity of MRSA. Epidemiology and characteristics of MRSA differ in different geographical regions. This study was conducted to characterize and determine the antibiotic susceptibility profile of MRSA strains isolated from patients in Hospital Tengku Ampuan Afzan, Pahang, Malaysia and to detect the presence of the *mecA* and *PVL* genes in the isolates. **Materials and methods:** In this study a total of 36 isolates of MRSA have been collected during a period of three months (1stFebruary -30thApril 2018). The susceptibility pattern of the isolates to ten different commonly used antibiotics were determined and the target genes were addressed by real-time PCR experiment. **Results:** Based on the identifying criteria, 44.4% of the isolates were CA-MRSA, and 55.5% were HA-MRSA. Resistance to oxacillin, cefoxitin and penicillin was 100%, gentamicin 88.8%, erythromycin 33.3%, tetracycline 77.7%, trimethoprim-sulfamethoxazole 61.1%, clindamycin 13.8%, chloramphenicol 11.1%, but no resistant strain of vancomycin was detected. Most of the isolates were resistant to more than three groups of antibiotics. Real-time PCR revealed that all the isolates were *mecA* positive and 4 isolates were *PVL*-positive. *PVL*-positive strains were CA-MRSA and susceptible to clindamycin. **Conclusion:** The study confirms multi-drug resistant MRSA in the study area, and shows that resistance to methicillin is *mecA* mediated. *PVL* carrier strains were present and related to CA-MRSA strains of the isolates.

KEYWORDS: MRSA, CA-MRSA, HA-MRSA, *mecA*, *PVL*

INTRODUCTION

Infectious diseases make globally the major cause of early deaths. Overgrowing rate of antibiotic-resistant bacteria have been threatening world population together with the recurrence of those infectious diseases which once had been considered

to be under the control mainly in developed countries. The recent drug resistance in bacteria has aroused big concern in the world.¹ *S aureus* is part of the normal flora of healthy individuals.² Toxins and exoproteins production is responsible for the pathogenic capability of *S aureus*.³ In addition, the prevalence of *S aureus* outside of the hospital is another great medical concern.⁴

Corresponding author:

Prof. Dr. Mohammed Imad A. Mustafa Mahmoud
Department of Basic Medical Sciences,
Kulliyah of Medicine,
IIUM Kuantan Campus, 25200 Kuantan,
Pahang, Malaysia
Tel No: +60-19 929 7407
Email: imad@iium.edu.my

Although, antibiotics have decreased the risk of *S aureus* infections, development of resistance to multiple antibiotics has challenged efforts to treat these infections easily and successfully.² *S aureus* firstly, became resistant to penicillin, the finding that caused the introduction of penicillinase-resistant penicillin such as methicillin and oxacillin to

treat infections caused by these resistant strains. MRSA was detected just two years after the introduction of methicillin in UK.⁴ MRSA strain of *S aureus* is defined as one that is resistant to the latter group of penicillinase-resistant “beta-lactams”.

Studies show that most of the MRSA strains are resistant to a wide spectrum of antibiotic groups, which are known as multiple-drug resistant MRSA.⁵ Vancomycin is used as the last option to treat MRSA, however, recently there are increasing reports from many countries on resistant to vancomycin.⁶ MRSA is implicated in causing the most common infections of the human body like skin infections, bone and joint infections and respiratory tract infections. Furthermore, MRSA also causes exotoxin-mediated infections. MRSA-induced infections are considered to cause long duration hospital stay, increased health care expenses, and increased the mortality rates.⁴ It has been stated that the improper use of antimicrobial agents is the key factor that helps with the spread of antibiotic-resistant bacteria.⁷ In MRSA, the main cause of the resistance to beta-lactam antibiotics are a modified protein named penicillin-binding protein 2a (PBP2a). This protein is encoded by the *mecA* gene and has lower affinity for penicillin.⁸

There are some staphylococci like *S vitulinus*, that has the *mecA* gene, but its presence is not associated with the resistance to beta-lactams. The gene of *mecA* that can cause the resistance to beta-lactams is located on a mobile genetic element called Staphylococcal Cassette Chromosome *mec* (*SCCmec*), this can be found in strains of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*.⁸ However, studies revealed that the resistance to methicillin may not only be mediated by the *mecA* gene.⁹ In addition, some strains of MRSA often carry Panton-Valentine Leukocidin (PVL) encoding gene.¹⁰

PVL acquired more importance in the near past because it had been linked with the CA-MRSA infections. PVL is an exotoxin of *S aureus* that causes apoptosis and has cytotoxic effects on the human neutrophils. *S aureus* strains harboring the PVL encoding gene rapidly spread and causing serious skin and soft tissue infections.¹¹ As the antibiotic susceptibility profile, genetic characteristics and

epidemiology of MRSA isolates differ in the different geographical area. Therefore, this study was conducted to define the susceptibility profile of MRSA isolates in the study area, and to look for the presence of *mecA* and *PVL* genes in these isolates.

MATERIALS AND METHODS

In this study, a total of 36 clinical MRSA isolates were collected over a period of 3 months (1stFebruary -30thApril, 2018). The sample was obtained from the Microbiology Laboratory, Pathology Department, Hospital Tengku Ampuan Afzan (HTAA), Pahang, Malaysia. The isolates originated from various types of patients' specimens. Patients were from different wards (surgical ward, orthopaedic ward, obstetrics and gynaecology ward, internal medicine ward, paediatric ward, outpatient department, intensive care unit, cardiology and skin wards). Patients' demographic and clinical data including (the history of hospitalization and surgical operation, medical history and chief complains, date of admission to hospital and the department where the patient was admitted), were collected from the hospital record book.

In this study CA-MRSA, HA-MRSA and MDR strains are defined as follows: CA-MRSA is strain, isolated from healthy individuals, patients in OPD (outpatients department), patients within 48 hours of hospitalization or from those patients who don't have the history of hospitalization or surgical operation during the last three months. HA-MRSA is strain isolated from hospitalized patients or from those who have a history of hospitalization and surgical operation during the last three months.¹² Multi-drug resistant strains are resistant to three or more groups of antibiotics.¹³ In this study *S aureus* ATCC 25923 (penicillin-susceptible strain), *S aureus* ATCC 29213 (penicillin resistant, beta-lactamase positive strain), and MRSA ATCC 35591 (positive for both the *mecA* and *PVL* genes) were used as a control or reference strains.

The isolates obtained from HTAA were re-identified and confirmed as MRSA by conventional phenotypic tests, including culture on blood agar, gram stain, catalase test, coagulase test, culture on mannitol salt agar, and resistant profile to oxacillin. Then the antibiotic susceptibility profile and genetic

characterization of the isolates were tested according to the research objectives. All the isolated strains were tested for antibiotic susceptibility by Kirby-Bauer disc diffusion method, in this test oxacillin (1µg), gentamicin (10µg), erythromycin (15µg), trimethoprim-sulfamethoxazole (1.25/23.75µg), chloramphenicol (30µg), tetracycline (30µg), vancomycin (30µg), ceftioxin (30µg), clindamycin (2µg) and penicillin's (10U) discs (Oxoid, UK) were used on Mueller-Hinton agar according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.¹⁴

As the disk diffusion test can only provide limited qualitative details of the susceptibility pattern of MRSA to vancomycin. Therefore, the minimal inhibitory concentration to oxacillin and vancomycin was evaluated by E-test (bioMerieux, USA) for all the isolates as well.

DNA Extraction: The DNA was extracted from the confirmed MRSA isolates, by using Presto™ Mini gDNA Bacteria Kit (Geneaid, USA). For this purpose, the MRSA strains were inoculated into 2.0 ml of Trypticase Soy Broth (TSB) and incubated at 37°C for 20 hours. Procedure for the DNA extraction was performed according to the manufacturer guidelines. The integrity and concentration of the extracted DNA were checked by NanoDrop and gel electrophoresis.

Real-time PCR: For detection of the *mecA* and *PVL* genes all the isolates were analysed by CFX96 Real-time PCR detection system (Bio-Rad, USA) using specifically synthesized primers, shown in the table I. The final volume of 25 µl of PCR reaction mixture contained 12.5 µl of GoTaq®, qPCR Mastermix (Promega, USA), 8.5 µl nuclease free water (Promega, USA), 2 µl of the template DNA and 1µl of each specific primer pairs. To justify the best annealing temperature for the system and primers, and to check the specificity of the primers, first, the system was run for the gradient test, then the Real-time PCR was run for each gene separately, according to the GoTaq® qPCR mastermix manufacturer's guideline with reaction conditions as explained in table II.

The same procedure and system were used for the detection of the *mecA* and *PVL* gene. The data was analysed by using Bio-Rad CFX Manager software (version 3.0) and melt curve. To confirm the results from real-time PCR and to make sure that the

amplified products were the target genes, the real-time PCR products were resolved by 1.5% agarose gel electrophoresis with 100 bp DNA ladder .

Table I: Specific primers for the *mecA* and *PVL* genes

Target gene	Sequence	Product size	Reference
<i>mecA</i>	F- TCCAGAT-TACAACCTCACCAGG R- CCACTTCATATCTT-GTAACG (Bioneer, Korea)	162 bp	¹⁵
<i>PVL</i>	F- TTCACTATTT-GTAAAAGTGTCAGACCC ACT R- TACT-AATGAATTTTTTATCGT AAGCCCTT (Bioneer, Korea)	180 bp	¹⁶

Table II: Real time PCR reaction conditions

Process	Cycles	Temperatures	Time
Hot-Start Activation	1	95C	2 minutes
Denaturation	40	95C	15 seconds
Annealing/ Extension		60C	60 seconds
Dissociation	1	60-95C	

RESULTS

In this study 36 MRSA isolates from patients of both genders and of different age groups were procured. The patients were admitted to different wards, including: surgical (6 patients), orthopaedic (5 patients), obstetrics and gynaecology (2 patients), paediatrics (2 patients), internal medicine ward (9 patients), outpatients department (3 patients), intensive care unit (3 patients), cardiology (5 patients) and skin ward (1 patient). The origins of the samples were blood (17 samples), sputum (2 samples), swabs (8 samples), pus (1 sample), soft tissue (3 samples), tracheal aspirate (4 samples) and bronchial lavage (1 sample). Based on the above-mentioned criteria in this study 16 out of 36 (44.4%) isolates were CA-MRSA strains and 20 out of 36 (55.6%) isolates were HA-MRSA strains. The results of the antibiotic susceptibility test by the disc-diffusion method and E-test (only for oxacillin and

vancomycin) are shown in table III. The antibacterial drug susceptibility test results in the table are categorized as resistant, susceptible and intermediate according to the CLSI guideline on the interpretation of inhibition zone diameters as stated in a study.¹⁴

In the E-test all the tested isolates were considered as oxacillin resistant, as their MIC was > 4.0 µg/ml. Conversely, all the tested isolates were considered as susceptible to vancomycin as their MIC was < 2 µg/ml. Multi-drug resistant groups of isolates are illustrated in figure I.

All 36 MRSA isolates were found to harbor the *mecA* gene as revealed by the real-time PCR. The presence of the *mecA* gene was confirmed by the melt curve analysis revealing a single peak and agarose gel electrophoresis showing the expected 162 bp amplicon band. Similar confirmation was done for the *PVL* gene resolving the 180 bp amplicon. All the PCR tests were done in triplicate. The *PVL* gene was detected in only 4 out of 36 isolates (14.4% of all tested isolates). The *PVL* gene positive strains were obtained from patients with localized soft tissue infections and sepsis, related to the CA-MRSA. There was no *PVL* positive strain in the HA-MRSA strains in this study.

DISCUSSION

S aureus is the most common isolated pathogen from the clinical specimens. Resistance to the available beta-lactam antibiotics has increased the clinical importance of this strain.¹⁷ Furthermore, the plethora of reports on multiple-drug resistant MRSA has escalated the alarm about MRSA infections. The emergence of CA-MRSA strains in a hospital setting had made it difficult to distinguish CA-MRSA from the HA-MRSA strains.¹² Moreover, the presence of the *PVL* gene in MRSA has added on concerns about this pathogen.¹⁸ Detection of *PVL* positive HA-MRSA has raised the concerns about the spread of higher virulence multi-drug-resistant strains of MRSA in hospitals.¹⁰ Published reports show the higher prevalence of *PVL* positive MRSA strains in the Asian countries.¹⁹ Looking at these findings this study addressed the antibiotic susceptibility pattern and genetic characterization of MRSA isolates in the study area. The majority of the MRSA isolates in this study were from patients in internal medicine wards. A similar finding was reported by Ghanznavi in a

study conducted in Kuala Lumpur Hospital, Kuala Lumpur, Malaysia.²⁰ Based on the criteria pointed by Chen,¹² in this study, 16 out of 36 isolates were CA-MRSA (44.4%) while the rest (55.6%) were HA-MRSA. However, the rate of CA-MRSA and HA-MRSA is different from area to area,¹² in a study the rate of HA-MRSA has been reported 59% from a tertiary teaching hospital in Malaysia.²¹ Previous studies have shown that the usual HA-MRSA strains had been spreading to the community while the common CA-MRSA strains had been spreading to the hospitals.²² Therefore, it might be difficult to consider a MRSA strain as a CA-MRSA or HA-MRSA based on the data collected from patients. In this study, most of the CA-MRSA strains were related to the skin and soft tissue infections. However, there are reports on vancomycin-resistant and intermediately resistant (VISA and heterogeneous VISA) strains of MRSA in Malaysia and elsewhere in the world.²³ In this study, vancomycin was found as the only antibiotic with no resistant strain to it, probably due to the strict control of its use in Malaysia.

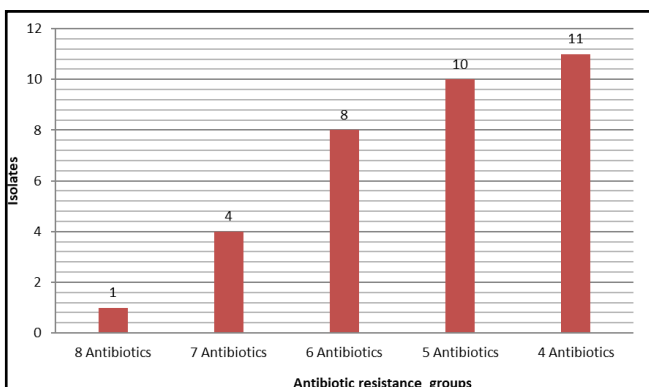
As the disc diffusion method can only provide limited qualitative information on the susceptibility pattern to vancomycin, in this study, we opted for the quantitative E-test but did not go further than that for detection of VISA and hetero VISA. In this regard, this study finding was concordant with that of the study conducted in a tertiary teaching hospital in Malaysia.²¹ This study shows that vancomycin is still the anti-staphylococcal drug option with the highest efficacy. This study found clindamycin as the second most effective anti-staphylococcal antibiotic among the tested antibiotics as 83.33% of isolates were susceptible to it. Others have also reported high rates of the susceptibility of MRSA to clindamycin.²⁴ This study found that all the *PVL* positive MRSA were susceptible to clindamycin. Resistance to chloramphenicol was also low (11.11%) but higher than to clindamycin. This study found that only a few of the tested strains were sensitive and intermediately sensitive to erythromycin (8.33% and 8.33% respectively). However, a study has reported full resistance to erythromycin in a tertiary hospital in Malaysia.⁵ Overall, the study findings show that most of the tested MRSA were resistant to multiple classes of antibiotics. In this study, we labeled the isolates which were resistant to three or more classes of antibiotics as multidrug-resistant, according to the study published by Saidi.¹³

Table III: The results of the antibiotic susceptibility test

Antibacterial drugs	Sensitive		Intermediate		Resistant	
	N*	%	N	%	N	%
Oxacillin (OX)	0/36	0%	0/36	0%	36/36	100%
Gentamicin (CN)	2/36	5.55%	2/36	5.55%	32/36	88.88%
Erythromycin (E)	3/36	8.33%	3/36	8.33%	30/36	83.33%
Trimethoprim-sulfamethoxazole (SXT)	12/36	33.33%	0/36	0%	22/36	61.11%
Chloramphenicol (C)	28/36	77.77%	4/36	11.11%	4/36	11.11%
Tetracycline (TE)	6/36	16.66%	2/36	5.55%	28/36	77.77%
Vancomycin (V)	36/36	100%	0/36	0%	0/36	0%
Cefoxitin (FOX)	0/36	0%	0/36	0%	36/36	100%
Clindamycin (DA)	30/36	83.33%	1/36	2.77%	5/36	13.88%
Penicillin (P)	0/36	0%	0/36	0%	36/36	100%

This study illustrates that the resistance pattern of MRSA strains varies so much among isolates from different patients so that prescription of antibiotic to MRSA infections based on the general background information cannot be the most effective approach. In this study, all MRSA isolates were characterized by real-time PCR. As dyes like SYBR® Green I may bind to unspecific amplified dsDNA²⁵, therefore, for higher confidentiality about the specific amplification, in addition to the analysis of the real-time PCR products by Bio-Rad CFX Manager software (version 3.0) and melt curve, the identity of the products were confirmed by gel-electrophoresis using a 100bp DNA ladder, as recommended by Broeders.²⁶

Figure 1: Multidrug-resistant isolates grouped according to the number of different antibiotics they resist



All those strains which were detected as MRSA by the phenotypic tests were detected as *mecA* positive. Similar findings have been reported from other Asian

countries.³ Similarly, a study conducted by Nezhad in Iran, has reported that all the isolates of MRSA, which had been confirmed as resistant to oxacillin on phenotypic base, harbored the *mecA* gene detectable by PCR.¹⁶ However, the prevalence of *PVL* positive MRSA differs geographically, overall, the Asian countries are reported to have a higher prevalence of *PVL* positive MRSA.¹⁰ In a European multicenter study, inter-country variation in *PVL* rates among MRSA strains has been reported to vary from 0% to 29%.²⁷ In our study 3 *PVL* positive strains were isolated from the patients with skin and soft tissue infections and one from a patient with sepsis.

All *PVL* positive strains were from CA-MRSA strains. This study shows a much lower percentage of *PVL* positive MRSA than the study finding in Nepal (56.8%).²⁸ The study in Nepal reported that 89.9% of the *PVL* positive strains were susceptible to clindamycin. The prevalence they have reported is much higher than the prevalence in this study, probably due to geographical variation. The *PVL* positive strains among MRSA isolates have been reported at a rate of 10.5% from a hospital in Turkey.²⁹ In Malaysia, the study published by San Sit reported 5.3% of *PVL* positive MRSA strains from a tertiary hospital.²¹ Another study from Malaysia reported 5.5% *PVL* positive MRSA strains.³⁰ Geographical and sample type and size differences might be the factors that explain the discrepancies in *PVL* rates.

CONCLUSION

This study explains the antibiotic susceptibility profile of MRSA strains to the commonly used anti-staphylococcal antibiotics in the study area. The study also illustrates that the resistance to methicillin is *mecA* mediated, and there are PVL positive strains of MRSA in the study area.

RECOMMENDATIONS

Prescription of antibiotics in MRSA infections should be based on proper antibiotic susceptibility test results. Regular studies on the genetic characterization of MRSA strains are needed in the study area to add knowledge about the epidemicity of MRSA strains and to detect the emergence of new strains with modified *mecA* gene in the study area. Furthermore, a study of the PVL gene in MSSA strains is also recommended in the same study area.

ETHICAL CONSIDERATION

Before carrying this study, all required approvals were achieved from the relevant committees and authorities as follows:

KRC : Kulliyah of Medicine Research Committee
IREC : IIUM Research Ethics Committee
NMRR : National Medical Research Register
MREC : Medical Research & Ethics Committee
CRC: Clinical Research Committee

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