

DST 001344

TAN SRI ABDUL MAJID LIBRARY
INTI INTERNATIONAL UNIVERSITY

RECONFIRMATION AND MOLECULAR CHARACTERIZATION OF MRSA &
MRSE ISOLATES

KUM SIAU LING

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF BIOTECHNOLOGY (HONOURS)

FACULTY OF HEALTH AND LIFE SCIENCES
INTI INTERNATIONAL UNIVERSITY
PUTRA NILAI, MALAYSIA

2017

NON-PLAGIARISM DECLARATION

By this letter I declare that I have written this thesis completely by myself, and that I have used no other sources or resources than the ones mentioned.

I have indicated all quotes and citations that were literally taken from publications, or that were in close accordance with the meaning of those publications, as such. All sources and other resources used are stated in the references.

Moreover I have not handed in a thesis similar in contents elsewhere.

In case of proof that the thesis has not been constructed in accordance with this declaration, the Faculty of Health and Life Sciences has the right to consider the research thesis as a deliberate act that has been aimed at making correct judgment of the candidate's expertise, insights and skills impossible.

I acknowledge that the assessor of this item may, for the purpose of assessing this item,

- reproduce this assessment item and provide a copy to another member of the University; and/or,
- communicate a copy of this assessment item to a plagiarism checking service (which may then retain a copy of the assessment item on its database for the purpose of future plagiarism checking).

In case of plagiarism the examiner has the right to fail me and take action as prescribed by the rules regarding Academic Misconduct practiced by INTI International University.

Kum Siau Ling

Name

I14006929

I.D.Number



Signature

27/4/2017

Date

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged, and completed under the supervision of Ms. Lalita Ambigai Sivasamugham and co-supervision of Dr. Geetha Subramaniam

Kum Siau Ling

Student ID: I14006929

27th April 2017

Ms. Lalita Ambigai Sivasamugham

(SUPERVISOR)

Dr. Geetha Subramaniam

(CO-SUPERVISOR if any)

ACKNOWLEDGEMENT

It had been a challenging and interesting journey for me to complete on this thesis. First of all, I would like to thank my family who have supported me along the way by providing me financial and emotion support. They are my strongholds that had given me the strength and motivation to continue in this pathway. Along the way, I would like to say thank you to my friends and classmates that supported me and comfort me whenever I stumbled.

Next, I would like to express my sincere appreciation to all lectures and staff, who have assisted me in this project. Special thanks to Dr Geetha Subramaniam, Dr Geeta Selvarajah, Dr Choong Chieh Wan, and Ms Emily Quek Ming Poh who had assisted me when I faced stumbling blocks in my project. I appreciated their act of kindness and willingness to share their knowledge, expertise and ideas with me whenever I seek help from them. Last but not least, a very special gratitude to my supervisor, Ms. Lalita Ambigai Sivasamugham, for guiding and assisting during this whole project. Thank you for being patient and encouraging me when I faced problems. I am thankful for all the effort and time to correct my work endlessly and for motivating me to improve my writing skills and techniques.

ABSTRACT

The treatment of diseases related to antibiotic resistance is becoming difficult due to the rapid development of antibiotic-resistant strains. Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) are some of the common antibiotic-resistant bacteria that are spreading rapidly in the hospitals and communities. The methicillin resistance of MRSA and MRSE is mainly due to the formation of penicillin binding protein 2a (PBP2a) encoded by *mecA* gene that is not inhibited by beta-lactam antibiotics. The *mecA* gene in MRSA is carried by staphylococcal cassette chromosome *mec* (SCC*mec*) and it classified into five major types; I-V. The aim of this study was to identify the types of SCC*mec* among the MRSA isolates, and to confirm the presence of *mecA* gene in the possible MRSE isolates as well as to determine the DNA extraction method that yield better quality and quantity of DNA. In this study, a total of 35 isolates isolated by Chuah (2016) from healthy individuals at INTI International University were analysed using confirmatory tests such as gram-staining, catalase test, MSA, Brilliance MRSA 2 Agar, and antibiotic susceptibility testing. Two DNA extraction methods; crude extraction and i-genomic BYF DNA Extraction Mini Kit were used in this study and compared. PCR was conducted on genomic DNA from possible MRSE isolates to detect the *mecA* gene. Furthermore, multiplex PCR was conducted on genomic DNA from confirmed MRSA isolates to identify the types of SCC*mec*. From the confirmatory tests, out of total 35 isolates, 4 were confirmed as MRSA and 6 isolates were possibly MRSE. This study also shows that i-genomic BYF DNA extraction Mini Kit yielded better quantity and quality of DNA than crude extraction based on the bands produced by agarose gel electrophoresis and the spectrophotometer analysis. Results from the PCR and SCC*mec* typing showed no amplification of *mecA* gene in all the possible MRSE isolates. Furthermore, the SCC*mec* types could not be identified from the MRSA isolates as no amplification of the specific genes. There are various factors could have contributed to the failure of detecting *mecA* gene and types of SCC*mec*.

TABLE OF CONTENT

	Page
NON-PLAGIARISM DECLARATION	ii
DECLARATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
TABLE OF CONTENT	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	x
CHAPTER	
1 INTRODUCTION	1
2 LITERATURE REVIEW	3
2.1 Antibiotic-Resistant Bacteria: MRSA & MRSE	3
2.1.1 Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA)	3
2.1.1.1 Community-associated MRSA (CA-MRSA)	6
2.1.1.2 Healthcare-associated MRSA (HA-MRSA)	7
2.1.2 Methicillin-resistant <i>Staphylococcus epidermidis</i> (MRSE)	7
2.2 Molecular Characterization of MRSA and MRSE	8
2.2.1 Pulsed-field Gel Electrophoresis (PFGE)	8
2.2.2 Multilocus Sequence Typing (MLST)	9
2.2.3 Staphylococcal Cassette Chromosome <i>mec</i> (SCC <i>mec</i>)	10
2.2.4 PCR-restriction Fragment Length Polymorphism (RFLP)	11
Typing	
3 MATERIALS AND METHODS	12
3.1 Bacterial Isolates	12
3.2 Preparation of Media	12
3.3 Preparation of Reagents	12
3.4 Growth of Isolates	12
3.5 Confirmatory Tests	13
3.5.1 Gram Staining	13
3.6.2 Mannitol Salt Agar (MSA)	13
3.6.3 Brilliance MRSA 2 Agar	13
3.5.4 Catalase Test	13
3.5.5 Antibiotic Susceptibility Testing- The Disc Diffusion	14
Assay	
3.6 Molecular Analysis	14

3.6.1	DNA Extraction	14
3.6.1.1	Crude extraction.	15
3.6.1.2	i-genomic BYF DNA extraction mini kit.	15
3.6.2	DNA Analysis	15
3.6.2.1	Agarose Gel Electrophoresis	15
3.6.2.2	Quantification and Purity Analyses	16
3.6.3	Polymerase Chain Reaction	16
3.6.3.1	The Detection of <i>mecA</i> Gene in Possible MRSE	16
3.6.3.2	SCC <i>mec</i> typing of MRSA isolates	17
3.6.4	Agarose Gel Electrophoresis of PCR Products	18
4	RESULTS	19
4.1	Isolation of Pure Cultures	19
4.2	Confirmatory Tests	20
4.2.1	Gram Staining	20
4.2.2	Catalase Test	20
4.2.3	Growth on Mannitol Salt Agar (MSA)	21
4.2.4	Growth on Brilliance MRSA 2 Agar	22
4.2.5	Antibiotic Susceptibility Testing- The Disc Diffusion Assay	22
4.3	Analysis of Extracted DNA	24
4.3.1	Conventional Method: Crude Extraction	24
4.3.2	i-Genomic BYF DNA Extraction Mini Kit	25
4.4	Polymerase Chain Reaction	26
4.4.1	The Detection of <i>mecA</i> gene in Possible MRSE Isolates	26
4.4.2	SCC <i>mec</i> Typing of MRSA Isolates	26
5	DISCUSSION	28
5.1	Confirmatory Tests	28
5.1.1	Gram Staining	28
5.1.2	Catalase Test	28
5.1.3	Growth on Mannitol Salt Agar (MSA)	29
5.1.4	Growth on Brilliance MRSA 2 Agar	29
5.1.5	Antibiotic Susceptibility Testing- The Disc Diffusion Assay	30
5.2	DNA Extraction	31
5.3	Polymerase Chain Reaction	32
5.3.1	The Detection of <i>mecA</i> gene in Possible MRSE Isolates	32
5.3.2	SCC <i>mec</i> Typing of MRSA Isolates	33
6	CONCLUSION AND RECOMMENDATIONS	35
	REFERENCES	37
	APPENDIX	43

LIST OF TABLES

Tables		Page
1	Diameter of zone of inhibition (mm) of Coagulase Negative Staphylococci (CoNS) treated with oxacillin	14
2	The primers used for amplification <i>mecA</i> from Sigma-Aldrich®	17
3	The volume of each components of PCR mixture to amplify <i>mecA</i> gene	17
4	The sets of primers used for SCC <i>mec</i> typing from Sigma-Aldrich®	18
5	The volume of each components used in multiplex PCR	18
6	Diameter of the zone of inhibition for oxacillin susceptibility testing (in triplicate)	23
7	The volume and concentration of reagents that were used to prepare cell lysis buffer	45
8	The amount of each component to prepare 10x TAE buffer	46
9	The volume and concentration of reagents that were used to prepare TE buffer	46
10	Appearance of colonies on nutrient agar	49
11	Results of the confirmatory tests and antibiotic susceptibility testing	51
12	The spectrophotometer reading, purity ratio and concentration of DNA extracted by crude extraction	53
13	The spectrophotometer reading, purity ratio and concentration of DNA extracted by i-genomic BYF DNA extraction Mini Kit	53

LIST OF FIGURES

Figures		Page
1	Cutaneous abscess on different body parts caused by MRSA	3
2	Severe form of MRSA infections	4
3	Number of deaths caused by both <i>Staphylococcus aureus</i> and Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA) from 1993 to 2012 in England and Wales	5
4	The different types of SCCmec element in <i>S. aureus</i>	6
5	PFGE gel of DNA fingerprints of MRSA and MSSA isolates that is <i>SmaI</i> digested	9
6	The morphology of colonies on nutrient agar	19
7	Gram-stained isolate A/2016 M /04 viewed under bright-field microscope at x1000 magnification	20
8	Isolate A/ 2016 M / 13 reacted with 3% (v/v) hydrogen peroxide to produce bubbles	21
9	Growth on Mannitol Salt Agar (MSA)	21
10	Growth on Brilliance MRSA 2 Agar	22
11	Antibiotic susceptibility testing- oxacillin disc diffusion	23
12	Agarose gel electrophoresis of DNA extracted by crude extraction	24
13	Agarose gel electrophoresis of the DNA extracted by i-genomic BYF DNA extraction Mini Kit	25
14	Agarose gel electrophoresis of PCR product from possible MRSE isolates.	26
15	Agarose gel electrophoresis of PCR product from SCCmec typing with set A primer	27
16	Agarose gel electrophoresis of PCR product from SCCmec typing with set B primer	27
17	Protocol for DNA extraction by i-genomic BYF DNA Extraction Mini Kit	47
18	DNA Ladders	48

LIST OF ABBREVIATIONS

A	Absorbance
AIDS	Acquired Immune Deficiency Syndrome
bp	Base pair
CRBSIs	Catheter-related bloodstream infections
CLSI	Clinical and Laboratory Standard Institute
CoNS	Coagulase Negative Staphylococci
CFU/mL	Colony forming units/millilitre
CA-MRSA	Community-Associated Methicillin-resistant <i>S. aureus</i>
°C	Degree Celsius
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dH ₂ O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
g	Gram
HA-MRSA	Healthcare-associated Methicillin-resistant <i>S. aureus</i>
hr	Hours
HIV	Human immunodeficiency virus
HCL	Hydrochloric acid
H ₂ O ₂	Hydrogen Peroxide
kb	Kilobase
L	Liter
MgCl ₂	Magnesium Chloride
Mg ²⁺	Magnesium ions
MSA	Mannitol Salt Agar

T _m	Melting temperature
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MRSE	Methicillin-resistant <i>Staphylococcus epidermidis</i>
MSSA	Methicillin-sensitive <i>Staphylococcus aureus</i>
μg	Microgram
μL	Microliter
μM	Micromolar
mL	Millilitre
mm	Millimetre
mM	Millimolar
min	Minutes
M	Molar
MDR	Multidrug resistance
MLST	Multilocus sequence typing
nm	Nanometre
OZ	Oxacillin
PVL	Panton-Valentine leucocidin
PBP	Penicillin binding protein
%	Percentage
PCR	Polymerase Chain Reaction
pH	Potential of hydrogen
PVE	Prosthetic valve endocarditis
PFGE	Pulsed-field gel electrophoresis
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minutes

RNA	Ribonucleic acid
s	Second
NaCl	Sodium Chloride
SCC <i>mec</i>	Staphylococcal cassette chromosome <i>mec</i>
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
UV	Ultraviolet
U	Units
V	Volt
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Staphylococcus aureus is a common microflora that can be found on the skin. However, some of the strains of *S. aureus* can cause infections that vary from mild conditions such as cutaneous abscess, to life-threatening bacteremia and toxic shock syndrome (Vysakh & Jeya, 2013). The treatment of diseases caused by *S. aureus* is becoming difficult due to the rapid development of antibiotic-resistant strains. One of such is the methicillin-resistant *Staphylococcus aureus* (MRSA) which is spreading rapidly in the hospitals and the communities (Vysakh & Jeya, 2013). Another common microflora of the skin is the *Staphylococcus epidermidis*. However, some strains are also found to cause hospital-acquired infection (Cherifi et al., 2013). Studies show that as many as 70% of *S. epidermidis* in the environment have been found to be resistant to methicillin (Miragaia et al., 2002). However, limited studies have been done on MRSE.

The methicillin resistance of MRSA is due to the formation of penicillin binding protein 2a (PBP2a) encoded by the *mecA* gene that is not inhibited by the beta-lactam antibiotics. The *mecA* gene is carried in the staphylococcal cassette chromosome *mec* (SCC*mec*) which is a mobile genetic element. This SCC*mec* enables the classification of MRSA into five major types (I-V) (Bhutia, Singh, Adhikari, & Biswas, 2015). Larger SCC*mec* elements types I, II and III (34-67kb) are usually carried by HA-MRSA whereas the smaller SCC*mec* element type IV and type V (21 to 25kb) are mainly found in CA-MRSA (Bhutia et al., 2015). Smaller SCC*mec* element (IV & V) carry genes that confer resistance only towards beta-lactam antibiotics, while larger SCC*mec* element (I, II, & III) have genes that confer resistance to multiple classes of antibiotics. This is because the latter (I-III) have additional integrated antibiotic resistant genes that enable them to resist many antibiotics (Eed, Ghonaim, Hussein, Saber, & Khalifa, 2015).

The SCC*mec* typing is used for epidemiological studies to investigate the types of MRSA in an environment and also to assist in the treatment process (Turlej, Hryniewicz, & Empel, 2011). This is because the results from the molecular typing can help to estimate the class of antibiotics that are ineffective against the respective MRSA

strains. In addition, this molecular typing method provides a rapid result compared to the tedious conventional method, the antibiotic susceptibility testing. Apart from that, molecular typing is also sensitive and provide reasonably accurate result to determine the most appropriate choice of antibiotic treatment for MRSA related diseases. Thus, molecular typing assay can be used as potential diagnostic tool in hospital.

Chuah (2016), isolated 35 possible MRSA and MRSE isolates from INTI International University. The MRSA isolates were confirmed using the Brilliance MRSA 2 agar but no study was done to determine the type of MRSA isolates and the possible MRSE isolates could not be further confirmed. Thus, this study was aimed to (1) Reconfirmation of MRSA & MRSE isolates through biochemical tests and antibiotic susceptibility testing. (2) Determine the types of SCC*mec* carried by the MRSA isolates isolated by Chuah (2016) using SCC*mec* typing and to (3) confirm the presence of *mecA* gene in the possible MRSE isolates. (4) This study was also to determine qualitatively the DNA extraction method that is more efficient in yield and quality of extracted DNA.

CHAPTER 2

LITERATURE REVIEW

2.1 ANTIBIOTIC-RESISTANT BACTERIA: MRSA & MRSE

2.1.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus is a common bacteria found on the skin and in the nostrils of human (Office of National Statistics, 2013). If *S. aureus* is able to enter into the body through the broken skin or other pathways, it can cause disease that varies from mild to life-threatening (Vysakh & Jeya, 2013). Most *S. aureus* infection are pyogenic, and some cause fever and local tissue destruction (Green et al., 2012). MRSA infections show the characteristics of pus production and pain at the site of infection and is often accompanied by swelling and inflammation (Green et al., 2012). Example of mild infection caused by MRSA are cutaneous such as an abscess (Figure 1).

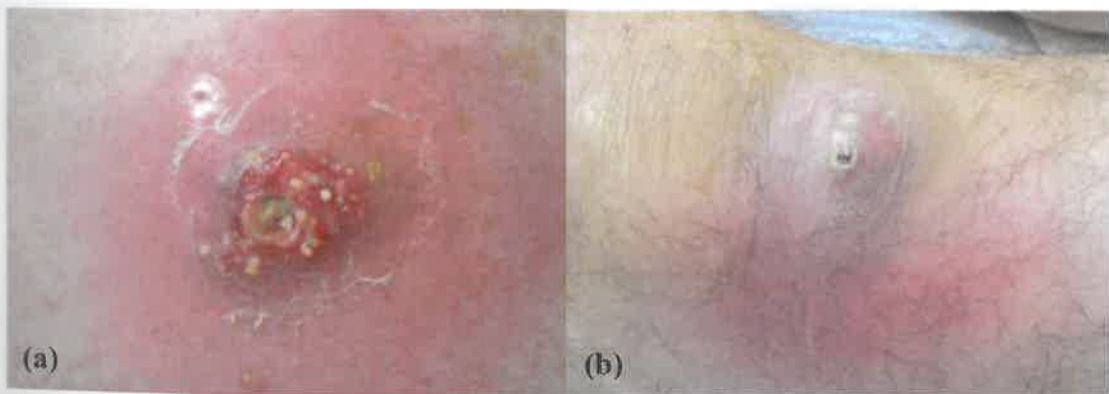


Figure 1. Cutaneous abscess on different body parts caused by MRSA. (Green et al., 2012)

Cutaneous MRSA wounds are frequently found on injured skin. These cutaneous MRSA wounds can lead to more serious complications such as cellulitis, erysipelas and folliculitis if the wounds are left untreated (Green et al., 2012). More severe form of MRSA infections include osteomyelitis, pyomyositis, purpura fulminans and necrotizing fasciitis (Figure 2)(Green et al., 2012). Bacteremia and endocarditis caused by MRSA are associated with relatively high mortality (Boucher, Miller, & Razonable, 2010). Studies also shows that HIV patients, burn victims and cancer