SCCmec TYPING OF MRSA AND MRSE ISOLATES FROM NILAI

VISALLINNE RAVICHANDAR

DISSERFATION SUBMITTED PARTIAL FULFILMENT OF THE , ... REQUIREMENTS FOR THE DEGREE OF BACHELOR OF BIOTECHNOLOGY (HONOURS)

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

MAY 2018

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.

| VISALLINNE RAVICHANDAR | MS LALITA AMBIGAI SIVASAMUGHAM |
|-----------------------------|--------------------------------|
| I15007352 | (SUPERVISOR) |
| 6 th August 2018 | |
| | (CO-SUPERVISOR if any) |

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my supervisor Ms. Lalita Ambigai Sivasamugham for the continuous support of my research, for her patience, motivation, enthusiasm and encouragement. Her guidance helped me in all the time of research in improving my skills set and writing of this thesis. I could not have imagined having a better supervisor for my study.

Besides my supervisor, I would like to thank my co-supervisor, Dr. Geetha Subramaniam, for her immense knowledge and insightful opinions every time I stumbled upon myself with questions. My sincere thanks also go to Dr. Wong Kok Kee and Dr. Geetha Selvarajah for offering me endless motivation and ideas on completing my research. I would also like to thank my fellow lab mate, Tan Weng Liang for the stimulating brainstorming sessions and words of encouragement during our time in the research laboratory and for all the fun we have had in the last three years. Also, I thank my friends, Pavethre Devan, Giitha Pucheearpah and Magdalen Rathimalar Michael.

Last but not the least, I would like to thank my parents Mr. Ravichandar Sinnasamy and Mrs. Umadevi Kuppusamy for supporting me financially, emotionally and spiritually throughout the completion of this study.

ABSTRACT

The presence of mecA gene in methicillin-resistant Staphylococcus aureus and methicillinresistant Staphylococcus epidermidis is the main reason for their resistance towards antibiotics derived from penicillin such as methicillin and cefoxitin. This gene encodes for penicillinbinding protein (PBP2a) which reduces the binding affinity of the bacteria towards β-lactam antibiotics. Previous studies show the isolation of numerous possible MRSA and MRSE isolates from healthy individuals in Nilai, as well as, from toilet door handles and handphones. However, only one isolate A/2016M/14 was identified to be mecA positive and of SCCmec type II. The other isolates were not investigated for mecA and SCCmec typing. Thus, this study was aimed to complete the molecular characterization of the remaining isolates through mecA amplification and SCCmec typing. Twenty-two out of thirty-two isolates were confirmed to be gram positive, clustered cocci with catalase production. These isolates were confirmed to be S. aureus and S. epidermidis by their growth on MSA. Eleven isolates of S. aureus and one S. epidermidis were found to be resistant to cefoxitin. Hence, these were confirmed as MRSA and MRSE isolates respectively. The DNA of these isolates was extracted using the cell lysis buffer and was subjected to mecA amplification using the MECA P4 and MECA P7 primers. Seven out of 12 isolates have the mecA gene and out of this, 6 were of SCCmec type II. This indicates the possible spread of a HA-MRSA clone within the community, as type II SCCmec is found in Hospital-acquired MRSA.

TABLE OF CONTENT

| | | | TABLE OF CONTENT | Page |
|-----|--------------------------|--|---|--|
| NO | N-PL | AGIAR | ISM DECLARATION | ii |
| DE | CLAI | RATION | · . | iii |
| AK | NOW | LEDGE | EMENT | iv |
| AB | STRA | ACT | | V |
| TA | BLE | OF CON | ITENT | vi |
| LIS | SŢ OF | TABLE | ES | viii |
| LIS | ST OF | FIGUR | ŒS | ix |
| LIS | ST OF | ABBRI | EVIATIONS | X |
| CH | APTI | ER | • | |
| 1 | INT | RODUC' | TION | 1 |
| 2 | 2.2 2.3 | Antibio 2.1.1 2.1.2 The Re Molect 2.3.1 2.3.2 2.3.3 2.3.4 2.3.5 | RE REVIEW Otic Resistant Staphylococci Methicillin-resistant Staphylococcus aureus (MRSA) 2.1.1.1 Hospital-acquired MRSA (HA-MRSA) 2.1.1.2 Community-acquired MRSA (CA-MRSA) Methicillin-resistant Staphylococcus epidermidis (MRSE) sistant Factors alar Characterization of MRSA and MRSE SCCmec typing (Staphylococcus cassette chromosome mec typing) spa typing Pulsed-Field Gel Electrophoresis (PFGE) PCR-restriction Fragment Length Polymorphism (RFLP) typing Multilocus Sequence Typing (MLST) | 3 3 4 4 5 5 5 6 8 8 9 |
| 3 | 3.1 3.2 3.3 3.4 | Prepara Growth Confirm 3.3.1 3.3.2 3.3.3 3.3.4 | ation of Media and Reagents of MRSA and MRSE matory Tests Gram-staining Catalase Test Growth on Mannitol Salt Agar (MSA) Disc Diffusion Test – Cefoxitin Analysis Extraction of DNA using Cell Lysis Buffer Agarose Gel Electrophoresis | 11 11 11 11 12 12 12 13 13 14 |

| | | 3.4.3 | Amplification of mecA Gene in MRSA Isolates | 14 |
|----|------|---------|---|----|
| | | 3.4.4 | SCCmec Typing of MRSA Isolates | 15 |
| | | 3.4.5 | Quantification and Purity Analysis | 16 |
| 4 | RES | ULTS | | 17 |
| • | 4.1 | | ılture Isolation · . | 17 |
| | 4.2 | Confirm | natory Tests | 17 |
| | | 4.2.1 | Catalase Test | 17 |
| | | 4.2.2 | Gram staining | 18 |
| | | 4.2.3 | Growth on Mannitol Salt Agar (MSA) | 19 |
| | | 4.2.4 | Disc Diffusion Test - Cefoxitin | 20 |
| | 4.3 | Agaros | e Gel Electrophoresis of Extracted DNA | 21 |
| | | 4.3.1 | Extraction of DNA using cell lysis buffer | 21 |
| | 4:4 | mecA (| Gene Amplification and SCCmec Typing in MRSA and MRSE | 22 |
| | , | 4.4.1 | Amplification of mecA Gene in Possible MRSA Isolates | 22 |
| | | 4.4.2 | SCCmec Typing of Isolated MRSA | 24 |
| 5 | DISC | CUSSIO | N . | 25 |
| | 5.1 | | on of Pure Culture | 25 |
| | 5.2 | | matory Tests | 25 |
| | | 5.2.1 | Gram staining | 25 |
| | | 5.2.2 | _ | 26 |
| | | 5.2.3 | Growth on Mannitol Salt Agar (MSA) | 26 |
| | | 5.2.4 | Disc Diffusion Test - Cefoxitin | 27 |
| | 5.3 | | Analysis [/] | 28 |
| | | 5.3.1 | Extraction of DNA using Conventional Method | 28 |
| | | 5.3.2 | mecA Gene Amplification | 28 |
| | | | 5.3.2.1 Amplification of <i>mec</i> A Gene | 28 |
| | | 5.3.3 | SCCmec Typing in MRSA & MRSE | 29 |
| | | y | 5.3.3.1 SCCmec Typing of Isolated MRSA | 29 |
| 6 | CO | NCLUSI | ON | 31 |
| RI | EFER | ENCES | | 32 |
| Δl | PPEN | DICES | | 42 |

LIST OF TABLES

| Tables | | Page |
|--------|---|------|
| 1 | Diameter of zone of inhibition (mm) for Coagulase Negative Staphylococci (CoNS) and <i>Staphylococcus aureus</i> treated with cefoxitin | 13 |
| 2 | Primers used to amplify mecA gene, | 14 |
| 3 | PCR mixture for mecA gene amplification | 15 |
| 4 | Zhang and Oliveira primers used to amplify SCCmec in MRSA isolates | 15 |
| 5 | Preparation of PCR mixture | 16 |
| 6 i | Cycling conditions for Zhang primers | 16 |
| 7 | Cycling conditions for Oliveira primers | 16 |
| 8 | The concentration and volume of reagents used to prepare cell lysis buffer | 48 |
| 9 | The concentration and measurement of reagents used to prepare 10x TAE buffer | 48 |
| 10 | Characteristics of colonies on nutrient agar | 49 |
| 11 | Results for confirmatory tests: gram staining, catalase test and growth on MSA of possible MRSA and MRSE samples | 52 |
| 12 | Antibiotic susceptibility test using cefoxitin in triplicates on possible MRSA and MRSE samples | 54 |
| 13 | Absorbance reading at 260 nm and 280 nm, concentration of DNA and purity ratio of extracted DNA of MRSA and MRSE samples | 57 |

LIST OF FIGURES

| Figures | | Page |
|---------|---|------|
| 1 | Cutaneous abscess on the foot (A) and hip (B) caused by MRSA | 4 |
| 2 | Amplification of SCCmec types I to VIII analyzed using agarose gel electrophoresis. | 8 |
| 3 | PFGE patterns of MRSA isolates digested with <i>Sma</i> I and stained using ethidium bromide | 9 |
| 4 . | Disc diffusion test using cefoxitin; (A) cefoxitin disc and (B) negative control: empty disc impregnated with deionized water | 13 |
| 5 , | Homogenous growth of N25D (MRSA) and N18(2) (MRSE) on nutrient agar | 17 |
| 6 | Gram positive, clustered cocci of isolate N25D viewed with 1000x magnification | 18 |
| 7 | The gram staining reactions and morphology of the isolates used in this study | 18 |
| 8 | Vigorous bubble production upon H ₂ O ₂ addition to isolate N25D | 19 |
| 9 | Growth of isolate N20(1) (A) and isolate FA301(B)I (B) on mannitol salt agar | 19 |
| 10 | The number of mannitol fermentation by isolates on MSA | 20 |
| 11 | (A) Cefoxitin resistant isolate, S16B/A and (B) cefoxitin susceptible isolate, S6 on MHA | 20 |
| 12 | Antibiotic susceptibility of MSSE, MSSA and methicillin-resistant isolates towards cefoxitin | 21 |
| 13 | Agarose gel electrophoresis of DNA extracted using cell lysis buffer. | 22 |
| 14 | Agarose gel electrophoresis of PCR products of mecA amplification. | 23 |
| 15 | Percentage of mecA-positive and mecA-negative isolates | 23 |
| 16 | Agarose gel electrophoresis of PCR products of SCCmec typing. | 24 |
| 17 | Appendix D: Basic structure of various types of SCCmec elements | 58 |
| 18 | Appendix E: ExcelBand 100 bp DNA ladder (DM2100)(SMOBiO®) | 59 |

LIST OF ABBREVIATIONS

CFU Colony forming unit

G

HA-MRSA Hospital-acquired MRSA

Hrs Hours

H₂0₂ Hydrogen peroxide

MSA Mannitol salt agar

SCI4203 Methods and Skills in Research (course)

MRSA Methicillin-resistant Staphylococcus aureus

MRSE Methicillin-resistant Staphylococcus epidermidis

μg Microgram

μL Microliter

μM Micromolar

Ml Millilitre

Mm Millimetre

mM Millimolar

Min Minute

MHA Mueller Hinton agar

Nm Nanometre

n.d. Not determined

O₂ Oxygen

Rpm Rotation per minute

sp. Species

U/ μL Units per microlitre

Vol. Volume

v/v Volume per volume

CHAPTER 1

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* has been indicated to be one of the primary causes of skin infections. MRSA has also been reported to cause more severe infections such as sepsis and pneumonia (CDC, 2016). Methicillin-resistant *Staphylococcus epidermidis* (MRSE) causes bacteraemia and hospital-acquired infections (Huebner & Goldmann, 1999). Almost 700, 000 cases involving antimicrobial-resistant microbes like MRSA and MRSE are reported each year (Wooller, 2017).

The presence of *mec*A gene has been identified to be the main reason for the resistance of MRSA and MRSE because, this gene encodes for penicillin-binding protein (PBP2A) that reduces the binding affinity of bacteria towards β-lactam antibiotics (Mackenzie, Richardson, Missett, Wood & Groves, 1993) (Aklilu, Nurhardy, Mokhtat, Zahirul & Siti Rokiah, 2016). The staphylococcal cassette chromosome *mec* (SCC*mec*) element contains genetic components that are transmissible between similar or distinct species (Hanssen & Sollid, 2006). Thus far, 11 types of SCG*mec* elements (Types I to XI) have been identified in MRSA and MRSE (Liu, Chen, Peters, Li, Li, Xu & Shirliff, 2016). Types I, II and III are found in hospital-acquired MRSA (HA-MRSA) whereas, types IV and V are found in the community-acquired (CA-MRSA) isolates. Types I, II and III have large SCC*mec* elements comprising of multiple resistant genes thus, making these isolates to be resistant against a wide range of antibiotics compared to types V and IV which are only resistant against β-lactam antibiotics (Oliveira, Tomsz & Lencastre, 2004).

MRSA infections have resulted in high fatality rates globally (Chambers, 1988). Thus, rapid and accurate diagnosis is essential to save lives. Many ways are being used to identify the types of SCC*mec* element for the treatment of related-diseases. One of such way is through molecular characterization. SCC*mec* typing for instance, uses established sets of primers that amplifies specific regions in the SCC*mec* element eventually determining the types of the SCC*mec* element. Knowing the types of the SCC*mec* enables one to know the resistant trait

and helps the treating doctor to determine the appropriate antibiotics for the affected patients. In addition, the information obtained is also used to study the current spread of resistant traits within communities.

Chuah L. (2016) isolated MRSA from the axillae region of an healthy individual in Nilai, in which, Chia (2017) has identified the isolate, A / 2016 / M to have a Type II SCCmec element. Since it is of type II, isolate A / 2016 / M is a HA-MRSA. However, not all of the MRSA isolated from the axillae and nasal samples obtained by Chuah (2016) were amplified and categorized. In addition, more MRSA and MRSE were isolated from studies conducted by Himashi (2017), Erlies (2018), Margaret (2018) and Smyrna (2018). Thus, this study was aimed to complete the molecular characterization of all the remaining MRSA and MRSE isolates through mecA gene and SCCmec typing.

CHAPTER 2

LITERATURE REVIEW

2.1 ANTIBIOTIC RESISTANT STAPHYLOCOCCI

2.1.1. Methicillin-Resistant Staphylococcus aureus (MRSA)

Staphylococcus aureus is usually found in the nasal cavity, on the skin and axillae but it generally does not pose any harm (Baron, 1996). However, some strains are gaining attention due to their resistance towards antibiotics (Lowy, 2000). One such pathogen is methicillin-resistant Staphylococcus aureus (MRSA) which is resistant towards several antibiotics including methicillin and penicillin (Coughlan et al., 2013) (Lowy, 2003).

MRSA is transmissible via direct contact with an infected individual, or an object that was handled by an infected person (Lights and Solan, 2017). A MRSA-infected individual can be identified by primary symptoms such as high fever, swollen and painful boils and an infected area filled with pus (Figure 1). Infections caused by MRSA can also lead to sepsis and pneumonia, if not detected early or not treated effectively (Wendt *et al*, 2014).

Healthcare facilities, day-care units and schools that practice poor sanitary habits are at risk of MRSA outbreaks (DeNoon, 2007) since the bacteria can thrive among people harbouring this pathogen. In addition, the people associated in these places might be the ideal carriers for the pathogen to be transmitted from one to another. The estimated MRSA infections throughout United States has increased to more than 80 000 cases of severe infections, in 2017, where 45% of the reported cases have resulted in death (National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2018). CDC (2016) also stated that healthy individuals may not know that they are carriers of MRSA and MRSE. Hence, they could spread these pathogens to others, as well as, to the environment. A recent study conducted by CDC (2017) has concluded that one in three healthy individuals who carry MRSA do not exhibit any symptoms. A study by both Chuah (2016) and Chia (2017) also supported this finding as MRSA was isolated from the skin of healthy individuals.

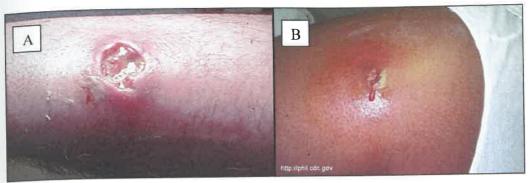


Figure 1. Cutaneous abscess on the foot (A) and hip (B) caused by MRSA (CDC, 2016).

2.1.1.1. Hospital-acquired MRSA (HA-MRSA)

MRSA can be categorized into two main classes; hospital-acquired MRSA and community-acquired MRSA (CA-MRSA) (Cunha, 2009). Hospital-acquired MRSA causes severe health issues such as pneumonia and skin infections (Leiber *et al.*, 2017; Hidron et al., 2008). Lights and Solan (2017) has stated that poorly maintained hospital linen and surgical tools causes the outbreak of HA-MRSA in hospitals. Apart from that, individuals who have undergone invasive surgery or have implantation of medical devices are also at risk of acquiring HA-MRSA infections that cause bacteremia (Nichols, 2017). According to the studies conducted by the American Academy of Family of Physicians (2014), healthcare workers were found to have improper hand sanitization and poor hygiene habits leading to numerous accounts of bacteremia and urinary tract infections caused by MRSA (Light & Solán, 2017).

2.1.1.2 Community-acquired MRSA (CA-MRSA)

CA-MRSA have been frequently isolated from healthcare facilities (Fukukawa et al., 2017) and recent studies have shown that infections caused by such bacteria are prevalent in these settings (Chua *et al.*, 2014). Generally, CA-MRSA causes soft tissue infections and skin infections among children, as well as, in adults (Maree *et al.*, 2007). Like HA-MRSA, poor hygiene has been the reason for the spread of CA-MRSA (American Academy of Family Physicians, 2014). CA-MRSA infections were also found to be prevalent in overcrowded settings such as, like in, army recruits training institutes, universities, gyms and prisons (Maree *et al.*, 2007). This is because, increased direct contact with unsanitized objects and sub environments such as gym equipments (Lights & Solan, 2017). A statistical study conducted by Statista (2017) in United States has mentioned that the number of individuals who opt to workout in the gym has increased from 32. 8 million in 2000 to 57.25 million in 2016. Therefore, more individuals end up sharing the same space and equipment in a gym. Thus, the

MRSA carriers may spread the pathogéns unknowingly across the room, increasing the chances of infection among more individuals (Lights & Solan, 2017).

2.1.2 Methicillin-resistant Staphylococcus epidermidis (MRSE)

Staphylococcus epidermidis is normally found on the mucosa and skin regions (Otto, 2013). They are also able to form biofilms on synthetic surfaces such as orthopaedic implants and intravascular catheters causing device-associated infections (Costa et al, 2018; Oliveira et al, 2018) and hospital-acquired infections (Zameer, Kreft & Gopal, 2008).

A study conducted by Li et al (2009), showed that most strains of *S. epidermidis* obtained from hospitals were resistant towards methicillin (Li et al., 2009) while some were resistant towards antibiotics like oxacillin, erythromycin, cefoxitin, penicillin and etc (Krishnaveni et al., 2013). Similar to MRSA, methicillin-resistant in *S. epidermidis* is caused by the presence of *mec*A gene (Cherifi et al., 2013).

In addition, *S. epidermidis* have higher resistance rates, thus making it more strenuous to eliminate (Cherifi et al., 2013). As according to Hussein & Rasheed (2016), almost 90% of *S. epidermidis* harbor the *mecA* gene which results in the high rate of methicillin-resistance among them.

2.2 THE RESISTANT FACTORS

According to Mackenzie et al (1993), the *mec*A gene is encoded by penicillin-binding protein (PBP)2a which leads to the resistance of MRSA and MRSE towards methicillin and β-lactam antibiotics. This protein reduces the binding affinity of the bacteria towards β-lactam antibiotics (Mackenzie et al., 1993). Regulation of *mec*A gene nonetheless is transcribed via *mec*RI, *mec*I, transmembrane β-lactam signal transduser respectively. The *mec*RI gene is activated in the presence of β-lactam antibiotics by the metallo-protease domain, facilitating the binding of PBP2A with *mec*A gene. Therefore, production of PBP2a increases and leads to the transcription of *mec*A (Deurenberg et al., 2007). The *mec*C is highly similar to *mec*A and is found to be encoded by a homolog of PBP2a (Ballhausen et al., 2014). Furthermore, five classes of *mec* complexes have been discovered thus far (Deurenberg et al., 2007).

Hanssen and Sollid (2016) stated that the SCCmec element contains genetic components that are transmissible between similar or distinct species and mecA gene. The gene is also

highly conserved (Ballhausen et al., 2014). Wisplinghoff et al, (2003) has distinguished major SCCmec types, for instance, types I to V that ranges from sizes 21kb to 67kb. However, types II and III are large in size (52kb and 68kb) explaining the resistance towards a wide range of antibiotics (Ma et al., 2002), whereas, types I, IV and V are relatively smaller (34kb, 21kb-24kb and 27kb) (Ma et al., 2002) and are resistant more specifically towards β-lactam antibiotics.

According to International Working Group of Staphylococcal Cassette Chromosome (n.d), *S. epidermidis* have been identified to have several new types of SCC*mec* (Types VII, IX, X and XI) (Ito et al., 2013). All eleven types of SCC*mec* elements present in MRSA (Appendix D). SCC*mec* type XI contains *mec*C gene conferring resistance towards heavy metals, β-lactam and non β-lactam antibiotics (Shore et al., 2011). SCC*mec* element types I, II, III and VIII are detected in HA-MRSA, whilst types IV, V, VI and VII are detected in CA-MRSA (Namvar et al., 2015). Even so, only types I to V are often times amplified in SCC*mec* typing of MRSA since they are acknowledged worldwide. The latter types are usually not amplified as the isolates with these genes have only been isolated from localized regions of the world and are not found globally (Vitali et al., 2014). However, the predominant strains in MRSE were identified to be SCC*mec* type III/ST2 and type IV/ST23 (Du et al., 2013). Both types have the ability to form biofilms and are resistant towards many antibiotics. A study conducted by Nomura et al., (2010) has identified that MRSE is not resistant towards antibiotics like minocycline, clindamycin and cephalosporin. Therefore, they can still be used to treat MRSE-related infections.

2.3 MOLECULAR CHARACTERIZATION OF MRSA AND MRSE

Molecular characterization studies are conducted to study the molecular basis of an organism (Mao, Hedrick & Chinchar, 1997). Furthermore, molecular characterization of MRSA and MRSE is done to identify chromosomal and DNA divergence that has occurred in these species by amplifying the *mec*A gene. Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is vital to understand the molecular characterization of MRSA. It is inevitable that molecular characterization has been very useful in the medical field. Reason being, it enables us to understand the existing widespread of certain diseases and thus, provide ways to treat the diseases with suitable drugs (World Health Organization, 2014). For instance, it has been identified that HA-MRSA carries SCC*mec* types I, II and III, and are resistant towards most antibiotics (Deurenberg *et al.*, 2007).