ASSOCIATION BETWEEN ANTIBIOGRAM AND MOLECULAR DETECTION OF *MECA* OR *MECC* GENE FROM METHICILLIN-RESISTANT COAGULASE NEGATIVE STAPHYLOCOCCAL SPECIES IN TWO GOVERNMENT HOSPITALS IN KUANTAN, MALAYSIA

BY

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ABSTRACT

In the last two decades, the emergence of nosocomial infections caused by CoNS has led clinicians and researchers to reconsider the role of CoNS and methicillin-resistant CoNS (MR-CoNS) as important agents of nosocomial infections. Because their natural habitat is skin, they may contaminate clinical samples causing suspicion as to whether an isolate is causing true infection or is a mere contaminant. The present study was conducted on clinical isolates of MR-CoNS obtained from inpatients in Tengku Ampuan Afzan Hospital (HTAA) and International Islamic University Malaysia Medical Center (IIUM-MC) to determine their antimicrobial resistance profile and the presence of *mecA* or *mecA* homologue (*mecC* gene). The isolates were cultured from clinical samples of blood, tissues, and swabs. A total of 40 isolates (33 blood, 4 tissues, and 3 swabs) of MR-CoNS were collected through venepuncture, biopsy, and swabbing techniques respectively, and processed by conventional cultural and biochemical methods, antimicrobial susceptibility tests, and finally confirmation to the species level was done by using conventional PCR assay for known four common clinical species. Of all 40 isolates, *Staphylococcus haemolyticus* was the most commonly found species (13/40, 32.5%), followed by *S. hominis* (12/40, 30%). *S. epidermidis* and *S. saprophyticus* were not identified in the samples. MR-CoNS isolated from blood included *S. haemolyticus* (12/40, 30%) and *S. hominis* (12/40, 30%), while 10 (40%) were unidentified. For swabs, only 1 (2.5%) isolate was identified as *S. haemolyticus*, and 1 (2.5%) was unidentified. For tissues, none of the 4 (10%) isolates tested positive for any of the 4 MR-CoNS. Methicillin- and vancomycin-resistance profile of the isolates was performed by E-test and broth micro-dilution methods. Of the 40 isolates, 38 were identified to be methicillin-resistant (MIC $\geq$ 0.5 µg/mL). The remaining 2 isolates were considered as susceptible to methicillin (MIC $\leq$ 0.25). All 40 isolates were found to be susceptible to vancomycin, with MIC ranging from 1-4 µg/mL. All 40 isolates were also tested for phenotypic antimicrobial susceptibility profile using the Kirby and Bauer disc diffusion method. Resistance rates to linezolid, erythromycin, ciprofloxacin, and ceftaroline were found to be 100%. Resistance rates to trimethoprim-sulfamethoxazole, teicoplanin, and clindamycin were found to be 82.5%, 92.5%, and 97.5% respectively. Thus, all the isolates revealed multi-drug resistance profiles to more than three antimicrobials, the highest being resistance to 9 antibiotics. All tested isolates showed multi-drug resistance profile to more than 3 antimicrobials except one isolate from swab. Detection of the *mecA* (or *mecC*) gene was performed by conventional PCR assay. Only *mecA* was identified in the 38 MR-CoNS isolates (95%). The other 2 isolates (5%) that were identified to be methicillin-sensitive by the E-test, also tested negative for the presence of the *mecC* gene, thus confirmed to be non-methicillin resistant. The high percentage of multi-drug resistance among these MR-CoNS isolates points toward the need for periodic antibiogram surveillance as they are identified to cause difficult to treat infections.

خلاصة البحث

في السابق، كانت تعتبرCoNS فقط microbiota العادية للجسم البشري التي يتم توزيعها على نطاق واسع وتزدهر على الجلد والأمعاء الأمامية وقناة الأذن الخارجية والجهاز التنفسي والأغشية المخاطية المعوية. ولكن في العقدين الماضيين، أدى ظهور الالتهابات المستشفوية التي تسببها CoNS للأطباء والباحثين إلى إعادة النظر في دور أنواع CoNS كعوامل مهمة للعدوى المستشفوية. ولأن موائلها الطبيعية هي الجلد ، فعادة ما يتم استردادها كملوثات للدم بدلاً من البكتيريا الحقيقية. وقد أجريت هذه الدراسة في مستشفى HTAA و IIUM-MC لتحديد ملامح المقاومة للميكروبات وتوصيف MR-CoNS المعزولة من العينات السريرية للمرضى الداخليين مثل الدم والأنسجة والمسحات. تم جمع 40 عزلة إكلينيكية (33 دم و 4 أنسجة و 3 مسحات) من MR-CoNS من خلال ثقب الوريد، خزعة، ومسح على التوالي، وتمت معالجتها بواسطة طرق الكيمياء الحيوية التقليدية، واختبارات الحساسية المضادة للميكروبات ، وأخيرا ، أكد باستخدام فحص PCR التقليدية. من بين 40 عزلة ، كان *S. haemolyticus* أكثر الأنواع شيوعا (13، 32.5 ٪)، تليها *S. hominis* (12، 30 ٪). لم يتم التعرف على *S. epidermidis* و *S. saprophyticus*. في عينة المختلف, في عينات الدم، كان 12 (30 ٪) من الدم الانحلالي، و 12 (30 ٪) *S. hominis*. بالنسبة للمسحات ، تم تحديد 1 (2.5٪) فقط من الحالة للدم. وبالنسبة للأنسجة، لا يوجد أي كائن حي تم التعرف عليه. تم بعد ذلك اختبار جميع العزلات 40 للتعرف على مظاهر الحساسية للمضادات الميكروبية باستخدام طريقة انتشار القرص. تم العثور على مقاومة للانزوليد ، الإريثروميسين ، سيبروفلوكساسين ، والسيفتارولين بنسبة 100 ٪. وقد لوحظت مستويات أخرى مثل 82.5٪ و 92.5٪ و 97.5٪ لتراي ميثوبريم سلفاميثوكسازول وتيكوبلانين وكليندامايسين على التوالي. تم إجراء تأكيد لمحات المقاومة للميثيسيلين والفانكومايسين بواسطة طرق E-test و microdilution. من بين 40 عزلة ، تم تحديد 38 عزلة لتكون مقاومة للميثيسيلين (حيث كان MIC الخاص بهم ≥0.5 ميكروغرام / مل). 2 المتبقية عرضة (كما كان MIC بهم 0.25 M). وحُددت جميع العزلات الأربعين لتكون عرضة للفانكومايسين (حيث تراوحت MIC من 1-4 ميكروغرام / مل). علاوة على ذلك، كشفت جميع العزلات عن ملامح مقاومة للأدوية المتعددة لأكثر من ثلاث فئات، أعلى مقاومة 9 فئات المضادات الحيوية. بشكل قاطع ، أظهرت 34 عينة دم ملامح المقاومة للعقاقير المتعددة لأكثر من 3 فئات ، تليها 4 عينات الأنسجة ، والأخيرة هي 2 عينات مسحة. كما تم تنفيذ تأكيد *mecA* (أو *mecC*) الجين بواسطة

الفحص PCR التقليدية. تم تحديد جين *mecA* فقط في 38 عزلة (95٪). في عينة المختلف، 32 (80 ٪) من العزلات كانت من الدم، و 4 (10%) كانت من الانسجة، و2 (5%) كانت من مسحات. أما العزلات الأخرى 2 (5٪) التي تم تحديدها لتكون عرضة للاختبار الإلكتروني ، وتم اختبارها بشكل أكبر لوجود تماثل ميكا (جين *mecC*) ، وأكدت أنها ليست مقاومة للميثيسيلين. تشير النسبة المئوية المرتفعة من المقاومة المتعددة للأدوية و جين *mecA* بين عزلات MR-CoNS هذه إلى الحاجة إلى المراقبة الدورية ، حيث تم تحديدها مؤخرًا على أنها تسبب صعوبة في علاج الالتهابات.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Master of Medical Sciences.

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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Signature …… Date ……..……..

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I dedicate this dissertation to my beautiful wife, Maryam Hussein Mayeye, and my lovely children, Muhammad bin Abdillahi and Ilham bint Abdillahi for their prayers, unconditional support and continuous encouragement throughout my coursework studies, and through the process of researching and writing of this dissertation. And also for their understanding and endurance while away. And to the memory of my lovely mother late Mawazo Twalib Mussa Kitota.

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LIST OF ABBREVIATIONS

&	and
≤	Less than or equal to
≥	Larger than or equal to
µ/mL	Microgram per millilitre
µL	Microliter
%	Percentage
mL	Millilitre
^{o}C	Degree Celsius
CFU/mol	Colony Forming Unit per mole
DNA	Deoxyribonucleic Acid
E-test	Epsilon test
Bp	Base pair
mecA	*mecA* gene
mecC	*mecC* gene
CLSI	Clinical and Laboratory Standards Institute
PCR	Polymerase Chain Reaction
TSB	Tryptic Soy Broth
mg	Milligram
Ng	Nanogram
MDR	Multi-drug resistance
MIC	Minimum Inhibitory Concentration
EtBr	Ethidium bromide
UV	Ultra-violet

IIUM	International Islamic University Malaysia
IIUM-MC	International Islamic University Malaysia Medical Centre
HTAA	Hospital Tengku Ampuan Afzan
CoNS	Coagulase-negative *Staphylococcus*
MR-CoNS	Methicillin-Resistant Coagulase-Negative *Staphylococcus*
SCC*mec*	Staphylococcal chromosomal cassete *mec*

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Staphylococcus aureus, also known as coagulase-positive staphylococcus (CoPS), has been the major *Staphylococcus spp* causing a host of classic and new syndromes and serious human diseases (Cunha et al., 2004). Hence, most studies on staphylococcal pathogenicity have focused on *S. aureus*, while little attention is paid to coagulase-negative staphylococci (CoNS) (Kim et al., 2018).

Till today, the definition of CoNS is based on diagnostic procedures that fulfil the clinical requirements of differentiating *S. aureus* that produce the coagulase enzyme and clot blood plasma, and those staphylococcal species that do not produce coagulase enzyme, and therefore, do not have the ability to clot blood plasma (Becker et al., 2014). Historically, CoNS were regarded as being harmless skin commensals or having little clinical significance and dismissed as culture contaminants because of their rarity in clinical pathology and the absence of virulence factors similar to those produced by CoPS (Argemi et al., 2019; Huebner et al., 1999; Becker et al., 2014). For-example, in the study by Robert et al. (1981), *S. epidermidis* recovered from blood culture was considered a contaminant.

However, in the last two decades, the emergence of nosocomial infections with CoNS has led clinicians and researchers to reconsider the role of CoNS species as important agents of nosocomial infections. Today, CoNS are well-recognised as causative agents of nosocomial infections, and the specific factors involved in their pathogenesis has been documented (Argemi et al., 2019; Deyno et al., 2018; Seng,

2017a; Becker et al., 2014). Following this recognition, frequently isolated CoNS species in clinical settings have been reported (Seng et al., 2017a; Sani et al., 2011; Klingenberg, 2007; Winderstrom, 2010). For example, Winderstrom (2010) reported that CoNS were responsible for about 30% of nosocomial infections. However, despite recognition of the clinical significance of CoNS in causing hospital-acquired infections by the end of the 1980's, most of their underlying molecular mechanisms have yet to be elucidated (Becker et al., 2014).

As normal microbiota of the human body, CoNS are widely distributed and thrive on skin, anterior nares, external ear canal, and the respiratory and gastrointestinal mucous membranes (Saber et al., 2017). It is reported that about half of CoNS species reside on healthy humans, and most of them are found abundantly on the skin, and are most frequently encountered by clinicians as contaminants of microbiological cultures (Sharma et al., 2001). It is also reported that CoPS colonizes nearly 30% of the human skin and gastrointestinal mucosae, whereas CoNS colonizes nearly all human skin (Argemi et al., 2019).

Even though CoNS are normal microbiota, they often become opportunistic etiological agents that prevail in numerous disease conditions to produce severe infections (Bannerman, 2003). Due to the development of interventional therapy, long-term hospitalization, and the increasing number of immunocompromised patients, these bacteria have become among the five most commonly reported pathogens in nosocomial infections, with *S. epidermidis* and *S. haemolyticus* being the most commonly isolated species (Deyno et al., 2018; Seng, 2017a; Becker et al., 2014; Murray, 2002). Currently, about 150 million intravascular devices are used every year in the US alone (Shah et al., 2013); and about 250,000 cases of CoNS blood stream infections have been estimated annually (Chen et al., 2017).

The mortality rate of these infections is about 1-25%, representing a great burden to the public health system (Chen et al., 2017). CoNS harbour staphylococcal chromosomal cassette *mec* (SCC*mec*) which is the mobile genetic elements that carries *mecA* and various antibiotic resistance genes, originating from methicillin-resistant *Staphylococcus aureus* (MRSA). The SCC*mec* elements harbour *mec* genes (methicillin resistant genes such as *mecA* or *mecC* genes) that provide resistance to methicillin and other antibiotics, such as beta lactams, aminoglycosides, and macrolides (Soumya et al., 2017). These species are often associated with infections in the old, in immune-compromised patients, as well as in premature babies fitted with medical device implants such as catheters and prosthesis (Cheung & Otto, 2010). The CoNS are capable of attaching themselves onto these devices because they can form biofilms, which are matrix-embedded bacterial clusters that attach to materials such as plastics and also to tissue surfaces (Longauerova, 2006). They are transmitted mainly by medical and/or nursing procedures.

Once medical appliances and devices are implanted inside the body of immunocompromised patients, they may be colonized by CoNS in different parts of the skin and mucous membranes of the host, acting as a source of endogenous infections (Becker et al., 2014). Due to their harbouring resistance genes such as *mecA* (or *mecC*), the success of the respective medical procedure is significantly compromised, resulting in enormous medical and economic burdens. The presence of *mecA* (or *mecC*) gene in CoNS has been widely associated with a high prevalence of methicillin/oxacillin-resistance among CoNS species to the extent that it has become a great public health concern.

The present study was conducted to determine the antimicrobial susceptibility profiles of MR-CoNS species isolated from infected patients in HTAA and IIUM-MC

against commonly used antibiotics in treating MR-CoNS infections, to identify common species by conventional PCR assays and the type of infections they cause, and to detect the presence of *mecA* (or *mecC*) gene responsible for methicillin resistance.

1.1.1 Statement of the Problem and Justification

As introduced earlier, in recent years, an increase in the number of methicillin/oxacillin-resistant coagulase-positive *Staphylococcus aureus* (MR-CoPS) and methicillin/oxacillin-resistant coagulase-negative staphylococci (MR-CoNS) strains have become a serious clinical and epidemiological problem, as resistance to this antibiotic implies resistance to all β-lactam antibiotics and possibly other antibiotics due to the ability to transfer resistance genes such as *mecA* (and its homologue, *mecC*), and other functional genes that are carried in the mobile genetic element known as the staphylococcal cassette chromosome *mec* (SCC*mec*).

Generally, MR-CoPS are more widely reported than MR-CoNS. For MR-CoNS, many studies have been carried out in South East Asia, Europe and North and South America including Mexico (Melendez et al., 2016), Brazil (Botelho et al., 2011), US (Sharma et al., 2001), Sweden (Widerstrom, 2010), London (Xu et al., 2018), Germany (Becker et al., 2004), West Indies (Akpaka et al., 2014), etc. In South East Asia, particularly in Thailand, Seng et al. (2017a; 2017b) reported biofilm formation in MR-CoNS and their high prevalence. However, these two reports studied environmental MR-CoNS isolates recovered from various hospital and community/university sites, and not clinical MR-CoNS isolates from patients.

Seng et al. (2017a) studied samples collected from the hospital environment such as patients' beds, intravenous poles, surgical and medical wards, medical trolleys, wash-basins, door handles, stethoscopes, nurse stations, the emergency room, the intensive

care unit, laboratory clothes, urinals, water taps, and toilets. Their other study (Seng et al., 2017b) studied samples collected from the university environment. This study reported a high prevalence of MR-CoNS from items such as library books, escalators and tables, restroom door handles, wash basin areas, urinary taps and toilets, canteen tables, bank notes and coins used for payment, ATM machines and water dispensers, computer rooms and items such as computer mice, earpieces, keyboards and power buttons, and outdoor surfaces such as handrails, exercise machines, and public buses.

In Malaysia, a study was conducted in Universiti Kebangsaan Malaysia Medical Centre (UKMMC), Bandar Tun Razak, Kuala Lumpur, identified *Staphylococcus epidermidis, S. saprophyticus* and *S. xylosus* from CoNS and MR-CoNS isolates using a multiplex PCR approach with primers specific for each species (Sani et al., 2011). However, the study did not identify the gene(s) responsible for methicillin/oxacillin resistance.

From the above background, it is clear that data concerning molecular characterization of MR-CoNS, particularly detection of *mecA* (or *mecC*) is scarce and to our knowledge, unavailable in Malaysia. For that reason, the present study is designed to fill this existing gap by determining the antibiogram and molecular detection of the *mecA* (or *mecC*) gene in MR-CoNS isolates collected in various wards of HTAA and IIUM-MC.

Therefore, the present study will provide important information about the antibiogram of MR-CoNS against antibiotics commonly used for their treatment in HTAA and IIUM-MC. It will also provide important knowledge on the MR-CoNS species harbouring *mecA* (or *mecC*) gene isolated from infected patients in HTAA and IIUM-MC.