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PHENOTYPIC AND MOLECULAR ANALYSES OF COAGULASE NEGATIVE STAPHYLOCOCCI, PREDOMINANTLY COMPRISING Staphylococcus epidermidis, ISOLATED FROM A STUDENT POPULATION IN MALAYSIA

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By

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September 2015

Chair: Assoc. Prof. Mohd. Nasir bin Mohd. Desa, PhD
Faculty: Medicine and Health Science

Coagulase negative staphylococci (CoNS), predominantly *Staphylococcus epidermidis*, are commonly considered as commensal skin and nasal colonizing bacteria but have emerged as one of the leading causes of nosocomial infections. To date, limited studies have been conducted to investigate CoNS or methicillin resistant CoNS (MR-CoNS) in Malaysian community. Hence, this study was initiated to evaluate the status of CoNS in relation to their nasal carriage, risk factors, antimicrobial resistance, molecular characteristics and genetic background of the bacterial collection in a student population. This study was conducted at Universiti Putra Malaysia in November 2013 involving 192 health science students. A self-administrated questionnaire on socio-demographic and risk factors was distributed, followed by collection of nasal swab. Cultivation on mannitol salt agar (MSA) plates and basic phenotypic tests were used to preliminarily differentiate CoNS from *Staphylococcus aureus*. All CoNS isolates were subjected to antibiotic susceptibility test against 10 antibiotics and *mecA* gene screening. The *mecA* positive isolates were further subjected to staphylococcal chromosome cassette *mec* (SCC*mec*) typing and multilocus sequence typing (MLST) to identify the SCC*mec* types and sequence type (ST) of the isolates, respectively. CoNS isolates that were resistant to at least one antibiotic or were *mecA* positive were subjected to species identification based on *tuf* gene sequencing, screening of erythromycin resistance associated genes (*ermA*, *ermB*, *ermC*, and *msrA*), random amplified polymorphic DNA- (RAPD) and enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). In this study, a total of 120 isolates of CoNS (62.5%) were collected from 192 health sciences students. Chi-square

test showed significant association of nasal carriage of CoNS with gender (P = 0.0455), history of cold or fever (P = 0.0147) and presence of unhealed wound (P = 0.0467). Similarly, the P-value of the use of intravascular devices was close to significant (P = 0.0589). The other risk factors (ethnicity, health status and habit of touching nose) were deemed as statistically not significant. Resistance to penicillin was the highest among the isolates (58.3%) followed by tetracycline (9.2%), cefoxitin (8.3%), erythromycin (7.5%), oxacillin (6.7%), ceftazidime (5.0%), gentamicin (2.5%) and rifampin (1.7%). Two isolates of CoNS were identified as multidrug resistant strains (MDR) which are resistant to more than three antimicrobial families. Among 76 isolates that were subjected to *tuf* gene sequencing, 73 isolates were identified as *S. epidermidis* and three isolates as *S. haemolyticus*. Fifteen isolates of CoNS (12.5%) harbored *mecA* gene (13 *S. epidermidis*; 2 *S. haemolyticus*) whereas five isolates (4.2%) carried *msrA* gene (3 *S. epidermidis*; 2 *S. haemolyticus*). Twelve of the *mecA* positive isolates carried SCC*mec* type IV (11 *S. epidermidis*; 1 *S. haemolyticus*), two isolates carried SCC*mec* type II (1 *S. epidermidis*; 1 *S. haemolyticus*) and one isolate carried SCC*mec* type I (*S. epidermidis*). MLST analysis of MRSE isolates revealed four STs: ST57 (n=6), ST59 (n=1), ST72 (n=1) and ST193 (n=1). However, four of the isolates had non-typeable (new) ST. RAPD and ERIC analyses revealed that majority of the isolates were largely genetically distinct. We observed a potential dissemination of a few distinct MR-CoNS carrying predominantly SCC*mec* type IV in the population rather than a clonal spread in both analyses. Overall, the study showed a high prevalence of nasal carriage CoNS which had relatively low level of antibiotic resistance except for penicillin and had extensive genetic diversity.

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sebagai memenuhi keperluan untuk ijazah Sarjana Sains

ANALISIS FENOTIPIK DAN MOLEKUL UNTUK NEGATIF KOAGULASE STAPHYLOCOCCI, TERUTAMANYA *Staphylococcus epidermidis*, YANG DIKUMPUL DARIPADA POPULASI PELAJA DI MALAYSIA

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Negative koagulase staphylococci (CoNS), terutamanya *Staphylococcus epidermidis* adalah mikrob yang biasanya hidup di permukaan kulit dan dalam rongga hidung tetapi telah menjadi penyebab utama bagi jangkitan mikrob di hospital. Sehingga kini, kajian mengenai CoNS atau ketahanan methicillin CoNS (MR-CoNS) terutamanya dalam kalangan komuniti di Malaysia kurang diberi prihatin. Maka, penyelidikan ini bertujuan untuk mengkaji taraf pembawa, factor-faktor risiko, ketahanan antimikrob, ciri-ciri molecular dan latar belakang genetik CoNS dalam kalangan populasi pelajar. Kajian ini dijalankan di Universiti Putra Malaysia pada November 2013 dengan melibatkan 192 pelajar Sains Kesihatan. Subjek kajian diberikan soal selidik yang mengandungi soalan mengenai sosio-demografik dan faktor-faktor risiko sebelum pengambilan sampel swab hidung. Pertumbuhan bakteria di atas agar garam mannitol (MSA) dan ujian asas fenotipik digunakan sebagai ujian awal untuk membezakan CoNS daripada *Staphylococcus aureus*. Semua isolat bakteria telah menjalani ujian kepekaan antibiotic terhadap 10 jenis antibiotic dan penyaringan gen *mecA*. Selanjutnya, sampel yang mempunyai gen *mecA* telah menjalani ujian pengelasan staphylococcal kromosom kaset *mec* (SCC*mec*) dan pengelasan jujukan multilokus untuk menentukan jenis SCC*mec* dan penjenisan jujukan (ST). Ujian-ujian seperti penjujuk gen *tuf* , penyaringan gen-gen penentang erythromycin (*ermA*, *ermB*, *ermC*, dan *msrA*), Amplifikasi DNA Polimorf Rawak (RAPD) and *Enterobacterial Repetitive Intergenic Consensus*-PCR (ERIC-PCR) dijalankan untuk mengenalpastikan ciri-ciri CoNS isolat yang menentang minimum satu jenis antimikrob atau mengandungi gen *mecA.* Sebanyak 120 CoNS isolat (62.5%) telah dikumpulkan daripada 192 pelajar Sains Kesihatan. Ujian khi-kuasa dua menunjukkan hubungan penting antara taraf pembawa CoNS dengan tiga faktor, iaitu jantina (P = 0.0455), jangkitan selesema atau demam (P = 0.0147) dan luka yang belum sembuh (P = 0.0467). Faktor-faktor lain seperti kaum, taraf kesihatan dan sebagainya dibuktikan tidak bererti tetapi faktor

penggunaan alat suntikan (P = 0.0589) dibuktikan mempunyai hubungan yang mungkin bererti. Dalam penyelidikan ini, taraf ketahanan terhadap penicillin adalah tertinggi (58.3%), diikuti oleh tetracycline (9.2%), cefoxitin (8.3%), erythromycin (7.5%), oxacillin (6.7%), ceftazidime (5.0%), gentamicin (2.5%) and rifampin (1.7%). Hanya dua isolat telah dikesan mempunyai ketahanan terhadap pelbagai dadah (MDR) iaitu tahan terhadap sekurang-kurangnya tiga kelas antimikrob. Daripada 76 isolat CoNS yang menjalani ujian penyaringan jujukan gen *tuf*, 73 isolat adalah *S. epidermidis* dan tiga isolat adalah *S. haemolyticus*. Lima belas isolat (12.5%) mengandungi gen *mecA* (13 *S. epidermidis*; 2 *S. haemolyticus*) dan lima isolat (4.2%) mempunyai gen *msrA* (3 *S. epidermidis*; 2 *S. haemolyticus*). Dua belas isolat yang positif *mecA* membawa SCC*mec* jenis IV (11 *S. epidermidis*; 1 *S. haemolyticus*), dua isolate membawa SCC*mec* jenis II (1 *S. epidermidis*; 1 *S. haemolyticus*) and satu isolat SCC*mec* jenis I (*S. epidermidis*). Melalui analisis MLST untuk isolate MRSE, sebanyak empat STs ditemui iaitu ST57 (n=6), ST59 (n=1), ST72 (n=1) and ST193 (n=1). Walaubagaimanapun, ST bagi empat isolat MRSE tidak dapat dijeniskan. Analisis RAPD dan ERIC menunjukkan bahawa kebanyakan isolat berbeza dari segi genetik. Kami telah memerhati potensi pembawa yang terhad terhadap beberapa MR-CoNS, terutamanya SCC*mec* jenis IV dalam kalangan pelajar ini berbanding penyebaran klon dalam kedua-dua analisis. Kesimpulannya, penyelidikan ini menunjukkan kadar pembawa CoNS yang tinggi di dalam hidung di mana ia mempunyai taraf antimikrob yang rendah (kecuali penicillin) dan kepelbagaian genetic yang ekstrem.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
AP-PCR	Arbitrarily primed polymerase chain reaction
ATCC	American Type Culture Collection
CNA	Colistin-nalidixic acid agar
CC	Clonal Complex
CLSI	Clinical and Laboratory Standards Instistue
ERIC	Enterobacterial repetitive intergenic concensus
FAME	Fatty acid modifying enzyme
GlcNAc	*N*-acetylglucosamine
IS	Insertion sequence
MGE	Mobile genetic element
MHA	Mueller Hinton agar
MLST	Multilocus sequence typing
MR-CoNS	Methicillin resistant coagulase negative staphylococci
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intercellular adhesion
PNAG	Poly-*N*-acetylglucosamine
PSM	Phenol-soluble modulin
RAPD	Random amplified polymorphic DNA
SCC	Staphylococcal Chromosome Cassette
SLV	Single locus variants
SPSS	Scientific Package of Social Science
ST	Sequence type
TSA	Trypticase soy agar
TSB	Trypticase soy borth
TSST-1	Toxic shock syndrome toxin 1

CHAPTER 1

INTRODUCTION

1.1 Introduction

Staphylococci are opportunistic pathogens that commonly colonize skin and mucous membrane of humans. The absence of enzyme coagulase distinguishes coagulase negative staphylococci (CoNS) from coagulase positive staphylococci, such as *Staphylococcus aureus*. *Staphylococcus epidermidis* is the most widespread CoNS species and is frequently isolated from human epithelia, axillae (armpits), head and nostril (Otto, 2009). The less frequently isolated species such as *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* are more commonly isolated from axillae and the female perineum area, respectively (Takeuchi et al., 2005). Generally, CoNS species distribute throughout the human body, indicating the importance of CoNS in maintaining healthy skin flora. The colonization of CoNS establishes on human skin almost immediately after birth and continues to be the dominant species during the first few months of life (Christensen & Brüggemann, 2014; Dong & Speer, 2014). CoNS usually maintain a mutual relationship with the human host by limiting the growth of pathogenic bacteria (Otto, 2014a). Despite the beneficial characteristics of CoNS, this bacterial species can cause infections in high risk individuals such as newborns, elder individuals and immunocompromised patients.

Two staphylococcal species, *S. aureus* and *S. epidermidis* have been associated with almost one million infections caused in hospitals every year around the world (Gill et al., 2005). Although CoNS have less pathogenic potential than *S. aureus*, the CoNS species particularly *S. epidermidis* and *S. haemolyticus* have emerged as the leading causative agents of nosocomial infections after *S. aureus* (Otto, 2009). CoNS infections are usually associated with the use of indwelling medical devices such as urinary tract catheters, pace maker, prosthetic joints and other medical implants (Ziebuhr et al., 2006). The lack of immunity barrier in high risk individuals eases the invasion and colonization of CoNS. The production of biofilm-associated proteins allows CoNS to adhere on inert surfaces and also host tissues. In addition to biofilm formation, clinical isolates of CoNS with multiple antibiotic resistances deteriorates the condition of nosocomial infections and increases the difficulty of treatments (Schoenfelder et al., 2010; Ziebuhr et al., 2006). Unlike methicillin resistant *S. aureus* (MRSA), eradication of antibiotic resistant CoNS particularly methicillin resistant strains is difficult because of the ubiquitous presence of CoNS and their role as commensal bacteria.

Multiple drug resistant (MDR) strains of CoNS which usually possess methicillin resistance, are not uncommon in clinical settings and are gradually emerging in

community settings. According to several studies, 60-85% of clinical strains are methicillin resistant CoNS (MR-CoNS) and 70-90% of hospital isolates of *S. epidermidis* are resistant to methicillin (MRSE), a rate which is actually higher than that of *S. aureus* (Otto, 2009; Jamaluddin et al., 2008). Moreover, the rate of MR-CoNS is relatively high in community settings (11-31%) (Lebeaux et al., 2012; Barbier et al., 2010; Ruppé et al., 2009; Jamaluddin et al., 2008; Silva, Mattos, Coimbra, Ferreira-Carvalho, & Figueiredo, 2001). Resistance to methicillin in staphylococci is due to the presence of the *mecA* gene which expresses mutated penicillin binding protein 2a (PBP2a), a transpeptidase with a low affinity for β- lactams (Zong, Peng, & Lu, 2011). The *mecA* gene is carried in mobile genetic elements (MGE) known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Zong et al., 2011). Due to the recombinases encoded by the cassette chromosome recombinase (*ccr*) gene, SCC*mec* complex can excise from and integrate into chromosomes among different staphylococcal species (Hanssen & Sollid, 2006). With flexible genomes, CoNS are believed to be the earlier reservoirs which transfer the methicillin resistance determinant into *S. aureus* to become MRSA (Otto, 2009; Hanssen, Kjeldsen, & Sollid, 2004).

In clinical settings, CoNS are usually considered as contaminants and are seldom identified to species level. However, with increased significance in infections, identification of CoNS species is important for better diagnosis and more efficient treatment. As compared to phenotypic identification methods, molecular methods involving polymerase chain reaction (PCR) and DNA sequencing are considered to produce more accurate results. The *tuf* gene sequencing which was found to have better discriminatory power than 16S rRNA, had been utilized in several studies to identify CoNS species (Bergeron et al., 2011; Hwang, Kim, Park, Song, & Kim, 2011; Heikens et al., 2005). Furthermore, genotyping methods such as random amplified polymorphic DNA-(RAPD) and enterobacterial repetitive intergenic consensus-PCR (ERIC) are useful in phylogenetic analysis of CoNS. These methods which are suitable for short term epidemiological studies can also be used to examine the occurrence of clonal dissemination in a community. On the other hand, multilocus sequence typing (MLST) is a relatively new genotyping method that analyzes phylogeny and evolution of bacterial species based on DNA sequencing of seven housekeeping genes. Currently, MLST is applicable for only *S. epidermidis* among CoNS species. However, with the advantages of standardized nomenclature and data comparison globally, this method has been utilized for many other bacterial species (Widerström, Wiström, Sjöstedt, & Monsen, 2012).

Community-acquired staphylococcal infections have been on the rise especially with increased antimicrobial resistance (Silva et al., 2001). Nonetheless, most research studies are concentrating on *S. aureus* or MRSA. Moreover, research on CoNS were usually focused on clinical isolates. There is limited information on the colonization of CoNS particularly antibiotic resistant strains in healthy individuals in community (Silva et al., 2001). Currently, there is only one report on clinical isolates of *S. epidermidis* in Malaysia and no research work on community acquired MR-CoNS have been reported. As a potential reservoir of

different antibiotic resistance determinants, CoNS can live as commensal microflora on human skin and can be potentially pathogenic to individuals with compromised immunity. Therefore, it is vital to investigate the dissemination of methicillin resistant strains among nasal isolates of CoNS from the community. Furthermore, genotyping analyzes can provide new insights and reveal the genetic relatedness between CoNS strains.

1.2 Problem statement

The prevalence rate of MR-CoNS in communities such as children, college students, public community and others from different countries was reported to be in the range of 11% to 31%. This raises the concern on the prevalent of nasal carriage CoNS especially potentially virulent strains in a student population. In addition, the antibiotic susceptibility patterns and the genetic background of CoNS collection from community were of great value for the literature as limited studies had been done on community isolates of CoNS.

1.3 Research hypothesis

It was hypothesized that there is high prevalence of nasal carriage CoNS in this student population. The prevalence rate of MR-CoNS was expected to be in range of 11 to 31% as reported in previous studies. The collection of nasal isolates CoNS was predicted to have a diverse genetic background.

1.4 Objectives

1.4.1 General objectives

In this study, our main objective was to investigate the phenotypic and molecular epidemiology of CoNS in a university student population. The status of CoNS was evaluated in relation to their nasal carriage and risk factors, antibiotic resistance patterns, molecular characteristics and genotypes.

1.4.2 Specific objectives

- To investigate the prevalence of CoNS nasal carriage within a student population and their association with socio-demographic and various risk factors.
- To examine the antibiotic susceptibility patterns of CoNS isolates through phenotypic methods (disc diffusion test).
- To identify the antibiotic resistance determinants such as *mecA* (SCC*mec*) and erythromycin resistance-associated genes (*ermA*, *ermB*, *ermC* and *msrA*) through PCR screening.
- To identify the representative CoNS isolates at the species level by *tuf* gene sequencing and to evaluate the genetic background of these isolates through different genotyping methods (RAPD-PCR, ERIC-PCR and MLST).

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