Species Identification of Coagulase Negative Staphylococci (CoNS) Isolates in Universiti Kebangsaan Malaysia Medical Centre (UKMMC)

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ABSTRACT

Coagulase negative Staphylococcus (CoNS) are common colonizers of the human skin but has become increasingly recognized as agents of clinically significant infections. At the Universiti Kebangsaan Malaysia Medical Centre (UKMMC), the prevalence of CoNS among *staphylococcus* genus in 2009 was 47.1%. The aim of this study was to identify *Staphylococcus epidermidis, S. saprophyticus* and *S. xylosus* from the CoNS and MRCoNS (methicillin resistant CoNS) collected in 2009 using a multiplex PCR approach with primers specific for each species. Our results showed that *S. epidermidis* is the most common species among both MRCoNS and CoNS isolates in UKMMC. Among 1142 CoNS strains, 68.4% were *S. epidermidis*, 1.3% were *S. saprophyticus* while 30.3% were non-typeable (other species). A total of 659 CoNS strains have been identified as methicillin resistant (MRCoNS); where 68.1% were *S. epidermidis*, 1.5% was *S. saprophyticus* and 30.3% were from other CoNS species. *S. xylosus* was not identified among the isolates.

INTRODUCTION

Coagulase negative *Staphylococci* (CoNS) are common colonizers of the human skin and was once considered relatively avirulent and probably a contaminant when isolated from clinical specimens. However, these organisms have become increasingly recognized as agents of clinically significant infections [1], especially in immunocompromised patients who have prostheses and other invasive medical devices in place [2]. Therefore, patients warded in burn units as well as neonatal and pediatric intensive care units have a high risk for CoNS infections [3].

Besides their pathogenicity, CoNS are also inherently resistant to antimicrobial agents like methicillin [4]. Methicillin resistant CoNS (MRCoNS) are clinically important as they are resistant to all β-lactam agents and other commonly used antimicrobial agents. Subsequently, an outbreak of MRCoNS has the potential of causing widespread antimicrobial resistance in a hospital setting, causing additional problems to patient management in terms of antimicrobial prescription. CoNS like *S. epidermidis* is more likely to be multidrug resistant compared to other CoNS species [5], therefore it has become increasingly important to rapidly identify these organisms for better patient management.

Two species of CoNS commonly associated with human infection are *S. epidermidis* and *S. saprophyticus. S. epidermidis* is well documented as a pathogen in cases of bacteraemia, prosthetic valve endocarditis, and prosthetic joint infections. *S. saprophyticus* is recognized as an opportunistic pathogen in urinary tract infections, particularly in young sexually active females. *S. xylosus* has very occasionally been identified as a cause of human infection and has been associated with pyelonephritis in humans.

As a pilot study into staphylococcal strains isolated in our university's medical centre, we collected index strains of *Staphylococcus sp*. isolated in 2009, and determined the prevalence of CoNS among *Staphylococcus* genus in our hospital. We also identified *S. epidermidis*, *S. saprophyticus* and *S. xylosus* from these isolates using a modified PCR protocol.

MATERIALS AND METHODS

BACTERIAL STRAINS

A total of 2426 staphylococcal related infections were recorded at the Universiti Kebangsaan Malaysia Medical Centre (UKMMC) in 2009. Index isolates from these cases were collected and established as strains. Strains that were identified as CoNS via coagulase and Dnase test were then stocked at -80°C in Brain Heart Infusion broth (Oxoid ltd, England) and 40% glycerol from time of isolation for further use.

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ANTIMICROBIAL SUSCEPTIBILITY TESTING

To determine the antibiogram of our collection of CoNS and identify MRCoNS among these isolates, antimicrobial susceptibility testing was performed for all collected strains using disk diffusion method on muller hinton agar (Oxoid ltd, England) according to Clinical Laboratory Standard Institute (CLSI) [6] recommendation. Antibiotics used in the test include penicillin, erythromyan, oxacilin, gentamicin, ciproflocaxin, mupirocin, fusidic acid, teicoplanin and vancomycin.

GENOMIC DNA EXTRACTION

Genomic DNA of tested CoNS was extracted using DNeasy Blood & Tissue Kit (Qiagen USA) according to the manufacturer's instructions. The concentration and purity of DNA was measured using Nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific, USA). Extracted DNA was stored at -20°C till further use.

CoNS SPECIES IDENTIFICATION

Species identification for our CoNS collections were carried out using a modified multiplex PCR protocol [7]. Briefly, specific primer pairs Se705-1 and Se705-2; Sap1 and Sap2; XYL-F and XYL-R for CoNS species *S. epidermidis*, *S. saprophyticus* and *S. xylosus* respectively were used to detect and differentiate between the species of tested strains. Nucleotide sequences of each primer are listed in Table 1. Multiplex PCR was performed under the following conditions: 4 min at 94°C; followed by 30 cycles of 2 min at 94°C, 1 min at 53°C, 2 min at 72°C; an extra annealing step of 5 min at 72°C and a final hold at 4°C. PCR products were analysed by electrophorhesis through a 1.5% agarose gel (Sigma) in 1 x TBE buffer (Figure 1). *S.aureus* was used as a negative control for each reaction.

Table 1. Nucleotide sequences of primers used in the study

Primers	PCR product size (bp)	Sequence (5'–3')	Annealing positions on Gene
Xyl-F	539	AACGCGCAACGTGATAAAATTAATG	1-25
Xyl-R		AACGCGCAACAGCAATTACG	539-520
Sap 1	221	TCAAAAAGTTTTCTAAAAAATTTAC	169-193
Sap 2		ACGGGCGTCCACAAAATCAATAGGA	379-355
Se705-1	124	ATCAAAAAGTTGGCGAACCTTTTCA	21-45
Se705-2		CAAAAGAGCGTGGAGAAAAGTATCA	145-121

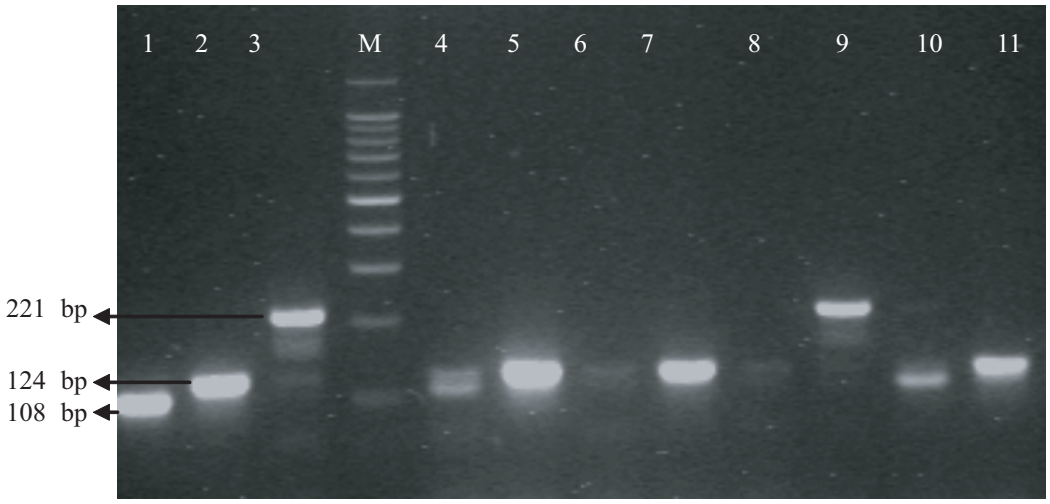

Figure 1. Multiplex PCR for CoNS species. Lane M, 100-bp molecular size DNA ladder (Promega); lane 1, negative control *S. aureus 81/108* (108bp); lane 2, positive control *S. epidermidis C.2* (124bp); lane 3, positive control *S. saprophyticus C.1334* (221bp); lanes 4-8 & 10-11, *S. epidermidis* isolates from patient; and lane 9, *S. saprophyticus* isolate from a patient.

RESULTS AND DISCUSSION

Coagulase and Dnase tests on 2426 staphylococcal strains used in this study revealed that 1142 of these strains were CoNS, giving a prevalence of 47.1%. From the MPCR, 68.4% of the strains were *S. epidermidis*, 1.3% were *S. saprophyticus*, while 30.3% were from other species. *S. epidermidis* was also the most prevalent species in MRCoNS (68.1%), followed by *S. saprophyticus* (1.5%) and other CoNS species (30.3%). On the other hand, some

studies identified *S. haemolyticus* as the most prevalent CoNS species in their hospitals [8, 9]. The predominance of *S. epidermidis* among CoNS and MRCoNS isolates found in our study is similar to the majority of previous studies [4,10,11,12,13,14]. This is not surprising, as *S. epidermidis* is the most ubiquitous normal flora of the human body [5] plus the fact that implants and prostheses are easily contaminated with this species if proper infection control and aseptic techniques were not adhered during surgical or implant procedures. In the study by Anne et al on 242 strains, as many as 67.4% strains were found to be *S. epidermidis*, while no *S. saprophyticus* isolate was detected [11].

Conventional phenotypic tests such as oxidation/fermentation of glucose in Hugh/Leifson medium, Voges-Proskauer reaction and susceptibility to novobiocin have long been established for the identification of Staphylococcus species [15,16]. However, these tests yielded false positive and false negative results frequently [15,16,17,18]. With the development of molecular techniques, specific nucleotide sequences could now be used as targets for molecular identification of *Staphylococcus* species. For example, Poyart et al. (2001) successfully developed a pair of degenerate primers that amplified an internal fragment of the staphylococcal *sodA* gene, which could then be sequenced to discriminate most of the Staphylococci [19]. Sequencing of the *hsp60 and rpoB* genes have also been reported to be useful for taxonomic and phylogenetic studies of Staphylococcus [20,21]. Nevertheless, these methods are time consuming and costly, as sequencing of the amplified products is required for species identification.

In order to simplify the process of species identification in Staphylococci, primers have been developed for species-specific detection of *S. aureus* [17,18,22], *S. epidermidis* [10,17], *S. saprophyticus* [23] and *S. xylosus* [16,17,24,25]. Some studies employed a multiplex PCR protocol that associates several primer pairs specific for various targets in the same amplification reaction. Martineau et al. (1998) developed a multiplex PCR assay which identifies *S. aureus* and *S. epidermidis* [18]; while Mason et al. (2001)'s protocol [22] could discriminate staphylococci from other genuses, distinguish *S. aureus* from CoNS and also identify oxacillin resistant staphylococci. In a separate research, Vannuffel et al. (1999) proposed a multiplex PCR-reverse hybridization approach targeting the *femA* gene in order to identify clinical staphylococcal species [26]. The researchers characterized a *femA* homologous gene in *S. epidermidis* and assessed the presence of *femA* homologous genes in *S. hominis* and *S. saprophyticus* by alignment of *S. epidermidis femA* sequences. For our study, we modified the multiplex PCR protocol from Morot-Bizot et al. [7] to identify and differentiate *S. epidermidis*, *S. saprophyticus* and *S. xylosus* in our collection of CoNS isolates. The protocol proved to be effective and time saving for CoNS identification.

In addition to species identification, antimicrobial susceptibility testing using various antibiotics prescribed in our medical centre was also performed for all CoNS. No isolate was found to be resistant to vancomycin; nevertheless, about 60% of the CoNS were resistant to commonly prescribed antibiotics such as erythromycin and oxacillin/methicillin. In addition, almost all of the strains (98.7%) were resistant to penicillin (Figure 2). CoNS have been documented to be inherently resistant to penicillins and cephalosporins [13,16], and there have been concerns that heavy, prolonged usage of these antibiotics in certain hospitals may select for multidrug-resistant commensal organisms such as methicillin resistant *Staphylococcus epidermidis* (MRSE). Therefore, proper antibiotic stewardship would be of crucial importance in medical centres recording a high percentage of multidrug-resistant CoNS.

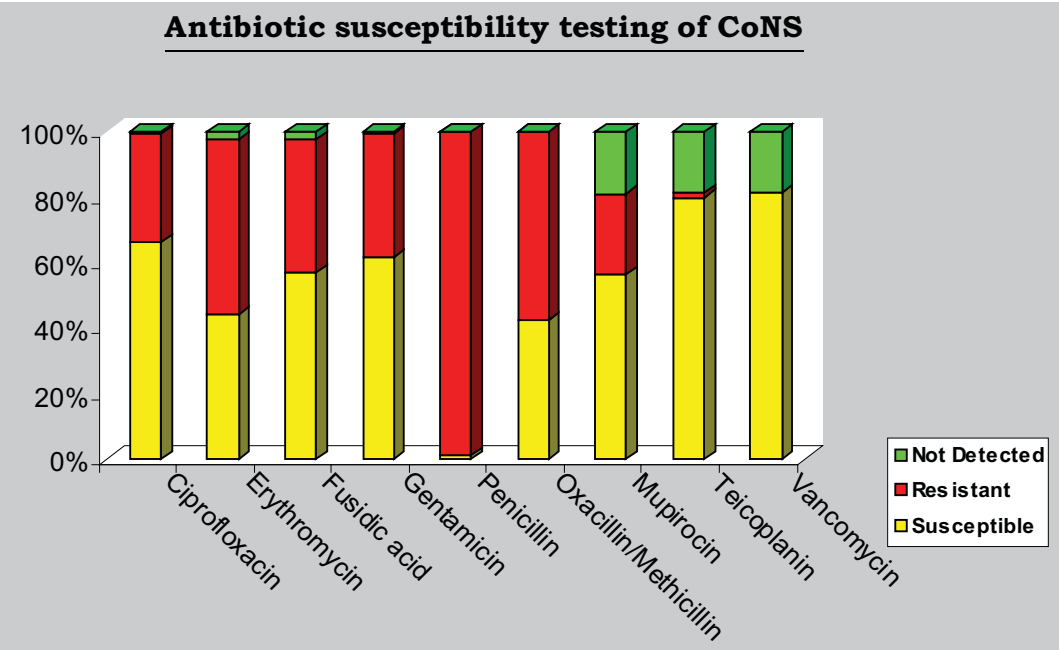

Figure 2. Antibiotic susceptibility testing for CoNS isolates used in the study

CONCLUSION

S. epidermidis was the most prevalent CoNS species identified in UKMMC in 2009; 57.7% of these isolates were methicillin resistant. As vancomycin is the standard treatment given for methicillin resistant *S. epidermidis* (MRSE), a high prevalence of MRSE might increase the selection pressure for vancomycin resistant *Staphylococcus* species and lead to limited treatment options in the future. Compared to conventional phenotypic tests, the multiplex PCR employed in this study is more accurate and rapid to perform, allowing CoNS species identification in atimely and reliable manner.

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REFERENCES

1. *Ryan KJ, Ray CG (editors). Sherris Medical Microbiology (4th ed.). McGraw Hill 2004. ISBN 0838585299.*
2. Almudena Rodrí guez-Aranda, Maria Daskalaki, Julia Villar, Francisca Sanz, Joaquí n, R. Otero and Fernando Chaves. Nosocomial spread of linezolid-resistant Staphylococcus haemolyticus infections in an intensive care unit. Diagnostic Microbiology and Infectious Disease's Journal 2009; 63: 398-402.
3. Weisman L. E. Coagulase-negative staphylococcal disease: emerging therapies for the neonatal and pediatric patient. CABI Abstract. 2002.
4. Walton D., Shields A., Reece K. and Bittner T. Comparison of Coagulase negative staphylococcus species identification using automated rep-PCR and *sodA* sequencing. American Society for Microbiology 2004; 104.
5. Kloos, W. Taxonomy and systematics of Staphylococci indigeneous to humans. In: Crossley KB, Archer GL eds. The Staphylococci in human disease. New York: Churchill Livingstone. 1997.
6. Clinical and Laboratory Standards Institute (CLSI): Performance Standards for Antimicrobial Susceptibility Testing. 16th Informational Supplement. CLSI document M100-S16. Wayne, PA 2006.
7. S. Corbiere Morot-Bizot, R. Talon and S. Leroy. Development of a multiplex PCR for the identification of Staphylococcus genus and four staphylococcal species isolated from food Journal of Applied Microbiology 2004; 97: 1087-1094.
8. Mehdinejad M, Sheikh AF, Jolodar A. Study of methicillin resistance in staphylococcus aureus and species of coagulase negative staphylococci isolated from various clinical specimens, Pak J Med Sci 2008; 24(5): 719-724.
9. Amita Jain, Jyotsna Agarwal and Seema Bansal. Prevalence of methicillin-resistant, coagulasenegative staphylococci in neonatal intensive care units: findings from a tertiary care hospital in India Journal of Medical Microbiology 2004; 53: 941-944
10. Martineau, F., Picard, F., Roy, P., Ouellette, M. and Bergeron, M. Species-specific and ubiquitous DNA-based assays for rapid identification of Staphylococcus epidermidis, Journal of Clinical Microbiology 1996; 34: 2888-2893.
11. Anne B. P., Robert S. and Lars V. P. Detection of methicillin resistance in Coagulase-negative staphylococci and in staphylococci directly from stimulated blood culture using the EVIGENE MRSA Detection Kit, Journal of Antimicrobial Chemotherapy 2003; 51: 419-421.
12. Huebner J., Goldman DA. Coagulase-negative staphylococci: role as pathogens. Annu Rev Med 1999; 50: 223-236.
13. PE Akpaka, N Christian, NC Bodonaik, MC Smikle. Epidemiology of Coagulase-Negative Staphylococcus isolated from clinical blood specimens at University Hospital of the West India. West Indian Med J 2006; 55(3): 170.
14. Kenneth L. Muldrew, Yi-Wei Tang, Haijing Li and Charles W. Stratton. Clonal Dissemination of Staphylococcus epidermidis in an Oncology Ward. Journal of Clinical Microbiology 2008; 46(10): 3391-3396.
15. Rossi, F., Tofalo, R., Torriani, S. and Suzzi, G. Identification by 16S-23S rDNA intergenic region amplification, genotypic and phenotypic clustering of Staphylococcus xylosus strains from dry sausages. Journal of Applied Microbiology 2001; 90: 365-371.
16. Morot-Bizot, S., Talon, R. and Leroy-Setrin, S. Development of specific PCR primers for a rapid and accurate identification of Staphylococcus xylosus, a species used in food fermentation. Journal of Microbiological Methods 2003; 55: 279-286.
17. Forsman, P., Tilsala-Timisjarvi, A. and Alatossava T. Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. Journal of Clinical Microbiology 1997; 143: 3491-3500.
18. Martineau, F., Picard, F.J., Roy, P.H., Ouellette, M. and Bergeron M.G. Species-specific and ubiquitous-DNA-based assays for rapid identification of Staphylococcus aureus. Journal of Clinical Microbiology 1998; 36: 618-623.
19. Claire Poyart, Gilles Quesne, Claire Boumaila and Patrick Trieu-Cout. Rapid and Accurate Species-Level Identification of Coagulase- Negative Staphylococci by using the sodA Gene as a Target. Journal of Clinical Microbiology 2001; 39(12): 4296-4301.
20. Anita Y. C. Kwok and Anthony W. Chow. Phylogenetic study of Staphylococcus and Macrococcus species based on partial hsp60 gene sequences. International Journal of Systematic and Evolutionary Microbiology 2003; 53: 87-92.
21. Michel Drancourt and Didier Raoult rpoB Gene Sequence-Based Identification of Staphylococcus Species. Journal of Clinical Microbiology 2002; 40(4): 1333-1338.
22. Mason, W.J., Blevins, J.S., Beenken, K., Wibowo, N., Ojha, N. and Smeltzer, M.S. Multiplex PCR protocol for the diagnosis of staphylococcal infection. Journal of Clinical Microbiology 2001; 39: 3332-3338.
23. Martineau, F., Picard, F.J., Menard, C., Roy, P.H., Ouellette, M. and Bergeron, M.G. Development of a rapid PCR assay specific for Staphylococcus saprophyticus and application to direct detection from urine samples, Journal of Clinical Microbiology 2000; 38: 3280-3284.

24. Aymerich, T., Martin, B., Garriga, M. and Hugas, M. Microbial quality and direct PCR identification of lactic acid bacteria and nonpathogenic staphylococci from artisanal low-acid sausages. Applied and Environmental Microbiology 2003; 69: 4583-4594.
25. Blaiotta, G., Pennacchia, C., Parente, E. and Villani, F. Design and evaluation of specific PCR primers for rapid and reliable identification of Staphylococcus xylosus strains isolated from dry fermented sausages. Systematic and Applied Microbiology 2003; 26: 601-610.
26. Pascal Vannuffela, Michel Heusterspreutea, Michèle Bouyerc, Bernard Vandercamb, Marianne Philippea, Jean-Luc Galaa. Molecular characterization of femA from Staphylococcus hominis and Staphylococcus saprophyticus, and femA-based discrimination of staphylococcal species. Res. Microbiol 1999; 150: 129-141.