Prevalence of methicillin resistance and macrolide–lincosamide–streptogramin B resistance in *Staphylococcus haemolyticus* among clinical strains at a tertiary-care hospital in Thailand

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Abstract

Staphylococcus spp. is a major cause of nosocomial infection and sepsis. However, increasing drug resistance is becoming a challenge to microbiologists. The purpose of this study was to identify and determine antimicrobial resistance phenotypes and drug resistance genes of clinical coagulase-negative staphylococci (CoNS) isolates at Mae Sot Hospital in Tak province, Thailand. A total of 229 CoNS isolates were collected from clinical specimens during two periods in 2014 and in 2015. *Staphylococcus haemolyticus* was the most prevalent species (37.55%), followed by *S. epidermidis* (21.83%), *S. saprophyticus* (11.79%) and *S. hominis* (11.35%) respectively. The remaining 17.48% of the organisms comprised *S. capitis, S. arlettae, S. cohnii, S. equorum, S. xylosus, S. warneri, S. sciuri, S. pettenkoferi, S. kloosii* and *S. lugdunensis*. Methicillin-resistant CoNS (MRCoNS), containing the *meca* gene, were detected in 145 of 229 isolates, mostly found in *S. haemolyticus* and *S. epidermidis*. In addition, the differentiation of their macrolide–lincosamide–streptogramin B (MLSB) resistance phenotypes was determined by the D-test and corresponding resistance genes. Among 125 erythromycin-resistant CoNS, the prevalence of constitutive type of MLSB, inducible clindamycin resistance and macrolide–streptogramin B resistance phenotypes were 72, 13.60 and 14.40% respectively. These phenotypes were expressed in 80% of MRCoNS isolates, mostly found in *S. haemolyticus* and *S. epidermidis*. In addition, the differentiation of their macrolide–lincosamide–streptogramin B (MLSB) resistance phenotypes was determined by the D-test and corresponding resistance genes. Among 125 erythromycin-resistant CoNS, the prevalence of constitutive type of MLSB, inducible clindamycin resistance and macrolide–streptogramin B resistance phenotypes were 72, 13.60 and 14.40% respectively. These phenotypes were expressed in 80% of MRCoNS strains. In addition, the *ermC* gene (79.20%) was found to be more prevalent than the *ermA* gene (22.40%), especially among MRCoNS. These results indicate that CoNS may play an important role in spreading of drug resistance genes. More attention to these organisms in surveillance and monitoring programs is needed.

Keywords: Coagulase-negative staphylococci (CoNS), macrolide–lincosamide–streptogramin B (MLSB) resistance, methicillin-resistant coagulase-negative staphylococci (MRCoNS), *Staphylococcus haemolyticus*

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**Introduction**

*Staphylococcus* is a bacterium in the family *Staphylococcaceae* and is a facultative anaerobe Gram-positive coccus (non–spore forming and nonmotile). It is normally found on human skin and causes a wide variety of diseases [1]. Besides *Staphylococcus aureus*, the coagulase-negative staphylococci (CoNS) are now recognized as important causes of human infection and are the most frequently isolated bacteria in the clinical microbiology laboratory. Its increasing importance is due to its role as a cause of hospital-acquired infection [2]. Among CoNS, *Staphylococcus haemolyticus* is the second most frequently isolated from human blood culture and is difficult to treat [3,4]. Other CoNS (i.e. *S. epidermidis, S. saprophyticus* and *S. lugdunensis*) remain a clinical challenge to human life and health [1]. One of the major challenges of routine microbiology is to identify and distinguish clinically significant CoNS from contaminant strains.

Evidence of multiple antibiotic resistance has been increasing in CoNS. Methicillin-resistant CoNS (MRCoNS) are found worldwide. They are associated with increased morbidity and
mortality \[1\]. Many genetic components are responsible for this resistance. The \emph{mecA} gene, which is not native to the \emph{S. aureus} genome (but has been transmitted between the \emph{S. aureus} strain and other staphylococcal species), is crucial to this development. These species include CoNS, i.e. \emph{S. epidermidis}, \emph{S. saprophyticus} and many other species \[4\]. Further, the rate of resistance to macrolide, lincosamide and streptogramin antibiotics among clinical staphylococcal isolates have increased and may confer cross-resistance to others \[5\]. Resistance to these antibiotics is mostly due to a target site modification by the methylation of ribosomal RNA, mediated by the presence of erythromycin resistance methylase (\emph{erm}) genes \[6\].

Phenotypic and genotypic identification has been developing to include biochemical testing for phenotypic identification and genotypic detection using nucleic acid–based methods, including specific gene amplification, PCR, 16S rRNA sequencing and mass spectrometry \[1\]. However, routine laboratory identification of \emph{Staphylococcus} spp. uses coagulase testing and biochemical tests to distinguish between \emph{S. aureus} and some other dominant staphylococcal species in the CoNS group (or all remaining staphylococcal species) and are classified as coagulase negative \[7\] in developing counties, and Thailand in particular. Biochemical tests are labor intensive and time-consuming. In addition, the use of commercial kits leads to higher costs.

The aim of this study was to establish the prevalence of methicillin and macrolide–lincosamide–streptogramin B (MLSA) resistance among various CoNS species isolated from clinical specimens using both phenotypic and genotypic approaches.

Materials and Methods

**Bacterial isolates**

The bacterial isolates were collected during two periods: March to June 2014, and May to September 2015. A total of 229 CoNS strains were isolated from different clinical samples (i.e. blood, sputum, urine, pus) at Maesot Hospital, Tak province, western Thailand. All isolates were initially identified using conventional biochemical tests. All bacterial isolates were stored in nutrient agar and delivered to the Department of Medical Technology, Naresuan University, Phitsanulok province. All isolates were obtained after receipt of approval from the human ethics committees of Naresuan University and Mae Sot Hospital. Species identification and antimicrobial susceptibility tests were carried out using standard laboratory methods \[8\] and followed the 2014 Clinical and Laboratory Standards Institute (CLSI) guidelines \[9\]. Ethical approval was obtained from the Naresuan University institutional review board (NU-IRB 269/58).

**Staphylococcal species identification**

All staphylococcal isolates were identified by phenotype to the species level according to morphology characteristics, Gram staining and catalase production after culture on a blood agar plate. First the Gram-positive cocci discrimination was done via the tube coagulase test, which discriminated \emph{S. aureus} from CoNS. Then several biochemical tests (with modified methods) were used to perform species classification: manitol salt agar, modified oxidase, novobiocin resistance, nitrate reduction, pyrrolidinyl arylamidase, urease test, nitrate reduction, ornithine decarboxylase, including carbohydrate fermentation (maltose, fructose, sucrose, lactose, \(\alpha\)-mannitol, \(\alpha\)-mannose, \(\alpha\)-trehalose and \(\beta\)-xylose) \[10,11\].

The discrepancy sample for strain classification were identified with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) at the Microbiology Laboratory, Department of Pathology, Ramathibodi Hospital, Bangkok. The 16S RNA sequencing for species confirmation was performed with \emph{Staphylococcus} genus—specific 16S rRNA for Staph756F (5\'-AAC TCT GTT ATT AGG GAA GAA CA-3\') and Staph750R 5\'-CCA CCT TCC TCC GGT TTG TCA CC-3\' \[12\] using an ABI 3730XLs sequencer (Macrogen, Korea).

**Phenotypic drug resistance detection**

All staphylococci isolates were tested by disk diffusion according to CLSI guidelines \[9\] against the following antibiotics, which were purchased from Oxoid (UK): oxacillin (1 \(\mu\)g), cefoxitin (30 \(\mu\)g), vancomycin (30 \(\mu\)g), clindamycin (2 \(\mu\)g), erythromycin (15 \(\mu\)g) and trimethoprim–sulfamethoxazole (25 \(\mu\)g).

**Methicillin resistance phenotype**

All CoNS isolates were detected for MRCoNS by cefoxitin disk. Briefly, bacterial suspension (approximately \(1 \times 10^8\) cells) was spread onto Mueller-Hinton agar and impregnated with a 30 \(\mu\)g cefoxitin disk (Hi Media, India). Then the plate was incubated at 35\(^\circ\)C for 16 to 20 hours. Any isolates with inhibition zone of \(<24\) mm were considered as MRCoNS, except \emph{S. lugdunensis}, which had the different cut point of \(<21\) mm \[9\].

**MLSA resistance phenotype**

To determine MLSA resistance, all isolates were tested for inducible clindamycin resistance by D-test as per CLSI guidelines \[9\]. A 0.5 McFarland bacterial suspension was cultured on Mueller-Hinton agar, impregnated with 2 \(\mu\)g clindamycin and 15 \(\mu\)g erythromycin disks (Hi Media) and were then placed in the centre of the plate 1.5 cm from the edges. Plates were incubated at 37\(^\circ\)C for 16 to 18 hours. The blunting of clindamycin zone of inhibition proximal to the erythromycin disk...
indicated as inducible clindamycin resistance (iMLS B), while resistance to both erythromycin and clindamycin indicated as constitutive type of MLS B (cMLS B); resistance to only to macrolide–streptogramin B but not to clindamycin was read as macrolide–streptogramin B (MSB) type [13].

Genotypic detection of resistance genes using multiplex PCR
Genomic DNA extraction. Staphylococcal DNA was extracted using the lysis method [14]. After pure bacterial incubation at 37°C for 24 hours in tryptic soy broth, the bacterial suspension was centrifuged at 3500 g for 15 minutes. The pellet was resuspended in 50 μL of lysostaphin (100 μg/mL in water; Sigma-Aldrich, USA). Cell suspensions were incubated at 37°C for 10 minutes; then 50 μL of proteinase K solution (100 μg/mL; Sigma-Aldrich) and 150 μL of buffer were added (0.1 M Tris, pH 7.5). The bacterial cell suspensions were incubated at 37°C for an additional 10 minutes and then placed in a boiling water bath for 5 minutes. They were then centrifuged at 12 000 g for 15 minutes, and the upper phase was transferred into a new tube. Chloroform/isoamyl alcohol mixture was added, and centrifugation was repeated using the same conditions. Lastly, pure ethanol was added to the tube and centrifuged. The pellet was air dried and suspended in sterile distilled water.

PCR assay for resistance genes. A multiplex PCR assay was used for targeting the mecA gene (a determinant of methicillin resistance), and the erm A, erm B and erm C genes (determinate of MLS B resistance). The oligonucleotide primers used in this study were synthesized and purchased from Macrogen. The following genotypes were used: MecA1 (5′-GTA GAA ATG ACT GAA CGT CCG ATA A-3′) and MecA2 (5′-CCA ATT C-3′) for mecA genes [15]; 5′-AAG CGG TAA ACC CCT CTG A-3′ and 5′-TTG TCA AC-3′ for erm A genes; 5′-CTA TCT GAT TGT TGA AGA AGG ATT-3′ and 5′-GGT TCG TTT AGG ATG AAA-3′ for erm B genes [16]; and 5′-CTT GTT GAC CAC GAT ATT TAC C-3′ and 5′-ATC TTT TAG CAA ACC CGT ATT C-3′ for erm C genes [17]. Two sets of PCR amplifications were performed in a ThermoCycler (Bio-Rad, USA) in 0.2 mL reaction tubes, each with a 25 μL reaction set (set A: mecA and erm C genes; and set B: erm A and erm B genes). Each mixture set was composed of 2× RBC blue Tag Mastermix (RBC Bioscience, Taiwan), primers and DNA templates. The amplification was monitored by electrophoresis on 1.7% agarose gel prepared in 0.5× TBE buffer and stained with ethidium bromide. The size of the amplified products was compared with 100 bp standard, and the gels were photographed under an ultraviolet transilluminator.

Results
Species identification of CoNS isolates
A total of 229 CoNS isolates were identified to species level by MALDI-TOF MS. The distribution of the sites of infection is shown in Table 1. The majority of the isolates were from blood (87.34%). Of 14 different CoNS species detected, S. haemolyticus had the highest prevalence (37.55%) and was the most frequent aetiologic agent found from blood culture samples. Other common CoNS species were S. epidermidis, S. saprophyticus, S. hominis and S. capitis. Several rare species, i.e. S. arlettae, S. cohnii, S. equorum, S. yfaosus, S. warneri, S. sciti, S. pettenkoferi, S. kloosii and S. lugdunensis, were also found.

| TABLE 1. Type of 229 specimens of coagulase-negative staphylococci isolates |
|--------------------------|-------------|----------------|----------------|----------------|----------------|----------------|----------------|
| Species                  | n (%) 2014 | n (%) 2015 | n (%) Total  | Blood (%)     | Body fluid (%) | Urine/Cath (%) | Sputum (%)     |
| S. haemolyticus          | 33 (35.11) | 53 (35.88) | 86 (37.55)   | 68             | 4              | 2              | 7              | 2              |
| S. epidermidis           | 30 (31.91) | 20 (12.60) | 50 (21.83)   | 45             | —              | 2              | —              | 2              |
| S. saprophyticus         | 4 (4.26)   | 23 (17.04) | 27 (11.79)   | 23             | —              | 2              | —              | 1              |
| S. hominis               | 13 (13.83) | 13 (9.63)  | 26 (11.35)   | 24             | —              | —              | 2              | —              |
| S. capsitis              | 4 (4.26)   | 7 (5.18)   | 11 (4.88)    | 11             | —              | —              | —              | 1              |
| S. arlettae             | NF          | 7 (5.18)   | 7 (3.06)     | 7              | —              | —              | —              | —              |
| S. cohnii               | NF          | 5 (3.70)   | 5 (2.18)     | 5              | —              | —              | —              | —              |
| S. equorum              | 4 (4.26)   | NF          | 4 (1.75)     | 4              | —              | —              | —              | —              |
| S. xylosus              | NF          | 3 (2.22)   | 3 (1.31)     | 3              | —              | —              | —              | —              |
| S. warneri              | 2 (2.13)   | 1 (0.74)   | 3 (1.31)     | 3              | —              | —              | —              | —              |
| S. sciti                | 2 (2.13)   | 1 (0.74)   | 3 (1.31)     | 3              | —              | —              | —              | —              |
| S. pettenkoferi         | 1 (1.06)   | 1 (0.74)   | 2 (0.87)     | 2              | —              | —              | —              | —              |
| S. kloosii              | 1 (1.06)   | NF          | 1 (0.44)     | 1              | —              | —              | —              | —              |
| S. lugdunensis          | NF          | 1 (0.74)   | 1 (0.44)     | 1              | —              | —              | —              | —              |
| Total                   | 94 (87.34) | 135 (11.70)| 229 (100)    | 200 (87.34)    | 4 (1.75)       | 6 (2.62)       | 10 (4.37)      | 21 (5.38)      |

NF, not found.
Phenotypic and genotypic characterization of MLSB and CoNS species

A total of 125 erythromycin-resistant isolates were phenotypically categorized as cMLSb, iMLSb or M5 by inducible clindamycin resistance (D-test). Among 125 erythromycin-resistant CoNS isolates, 100 (80%) were also resistant to methicillin. The cMLSb was found to be the most common phenotype (72%), while the iMLSb and M5 phenotypes had approximately similar prevalence (13.6 and 14.4% respectively). The ermC gene was the predominant gene among MLSB isolates (99/107). Among these isolates, 20 (20.2%) of 99 were found in combination with the ermA gene (28/107 = 26.17%). They also predominated in MRCoNS isolates. Notably, we study found the coexistence of ermA and ermC genes in erythromycin-resistant MRCoNS strains, while the ermB gene was not found in any strains (Tables 3 and 4).

Discussion

CoNS are now recognized as important causes of human infections, especially in hospital-acquired infections [18]. CoNS are also frequently isolated in clinical microbiology laboratories [4,19]. Understanding the possible sources of antimicrobial resistance determinants is important because they usually have a high prevalence of multidrug resistance. To our knowledge, this is the first study to determine the prevalence of CoNS species and their significant antibiotic resistances isolated from hospital patients in Thailand. There were 14 main species in the clinical specimens. It is noteworthy that S. haemolyticus was found predominantly among CoNS infections, especially in infections of the bloodstream. However, S. epidermidis, S. saprophyticus and S. hominis also showed a high incidence in infection site severity. Rare CoNS species were observed, i.e. S. capitis, which could be emerging opportunistic pathogens [20]. Further, S. lugdunensis has been reported to be a highly virulent CoNS species, and it is likely to be underreported [21,22]. The detection of S. haemolyticus as the most frequent species in our study could be related to many reports about the high prevalence of this organism in the hospital environment and its importance as a cause of hospital-acquired infection [3,20]. This infection can also affect medical personnel as well as patient and hospital support staff, who may be either transient or permanent residents. Therefore, it is important to characterize and distinguish among S. aureus strains and CoNS species, including drug susceptibility, even if it is costlier and requires additional time, unless automated identification systems are available.

In addition, the CoNS strains also demonstrated resistance to antimicrobial drugs, as seen in S. aureus. High incidence of methicillin resistance via the mecA gene was detected in up to 80.23% of S. haemolyticus, and the most species contained this gene (69/229 = 30.13%). Moreover, the resistance to clindamycin may occur during treatment, especially in erythromycin-resistant strains. In our study, the cMLSb-resistant phenotype

| Species (n)          | mecA-mediated oxacillin resistance | mecA gene |
|----------------------|-----------------------------------|-----------|
| S. haemolyticus (86) | 69                                 | 69        |
| S. epidermidis (50)  | 22                                 | 22        |
| S. saprophyticus (27) | 12                               | 12        |
| S. hominis (26)      | 13                                 | 13        |
| S. capitis (11)      | 9                                  | 9         |
| S. arlettae (7)      | 5                                  | 5         |
| S. cohnii (5)        | 4                                  | 4         |
| S. equorum (4)       | 2                                  | 2         |
| S. xylosus (3)       | 3                                  | 3         |
| S. women (3)         | 2                                  | 2         |
| S. sciu (3)          | 1                                  | 1         |
| S. pettenkafieri     | 1                                  | 1         |
| S. kloosi (1)        | 1                                  | 1         |
| S. lugdunens (1)     | 1                                  | 1         |
| Total (125)          | 145 (63.32%)                       | 145 (63.32%) |

CoNS species, and it is likely to be underreported [21,22]. The detection of S. haemolyticus as the most frequent species in our study could be related to many reports about the high prevalence of this organism in the hospital environment and its importance as a cause of hospital-acquired infection [3,20]. This infection can also affect medical personnel as well as patient and hospital support staff, who may be either transient or permanent residents. Therefore, it is important to characterize and distinguish among S. aureus strains and CoNS species, including drug susceptibility, even if it is costlier and requires additional time, unless automated identification systems are available.

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| Species (n)          | Drug resistance genes |
|----------------------|-----------------------|
|                      | mecA | ermA alone | ermC alone | ermA + ermC |
| S. haemolyticus (54) | 49   | 2          | 34         | 10         |
| S. epidermidis (28)  | 14   | 2          | 14         | 6          |
| S. saprophyticus (11)| 10   | 0          | 7          | 2          |
| S. hominis (8)       | 7    | 0          | 7          | 0          |
| S. capitis (8)       | 7    | 2          | 4          | 1          |
| S. arlettae (4)      | 4    | 0          | 4          | 0          |
| S. cohnii (4)        | 3    | 1          | 3          | 0          |
| S. equorum (3)       | 1    | 0          | 2          | 1          |
| S. xylosus (1)       | 1    | 0          | 1          | 0          |
| S. women (1)         | 1    | 0          | 1          | 0          |
| S. sciu (1)          | 1    | 0          | 1          | 0          |
| S. pettenkafieri (1) | 1    | 1          | 0          | 0          |
| S. lugdunens (1)     | 1    | 0          | 1          | 0          |
| Total (125)          | 100  | 8 (6.4%)   | 79 (63.2%) | 20 (16%)   |
was significantly higher among MRCoNS strains; this finding was in accordance with previous studies from Turkey [13,23]. Notably, they were also mostly found in *S. haemolyticus* and *S. epidermidis* (data not shown). Furthermore, we detected 17 (13.6%) of 125 CoNS isolates (iMLS$_B$ phenotype) to be erythromycin resistant and clindamycin susceptible by D-test. This suggests that 13.6% of these isolates would be falsely evaluated as susceptible to clindamycin if we had not done the D-test.

The prevalence of *erm* genes may vary according to geographical region or with the variable use of erythromycin. In this study, the incidence of *ermC* was greater than that of *ermA* genes among CoNS MLS$_B$ strains, especially in MRCoNS, as seen in a Korean study [5], whilst the *ermB* gene was not seen in any CoNS isolates. These findings echo that of studies done in Italy and Turkey [23,24]. Although this study did not find the *ermB* gene in any CoNS isolates, it supports the lower incidence rate of finding the *ermB* gene in CoNS isolated from humans [25]. A notable finding of the present study was the coexistence of *ermA* and *ermC* genes in MRCoNS strains, as previously studied in methicillin-resistant *S. aureus* and MRCoNS [5,23]. Because *S. haemolyticus* and some species of CoNS have a tendency to multiple antibiotic resistance, and because many clinical specimens indicate their presence, they may become a serious public health problem [3].

Our study reiterated CoNS species identification from clinical specimens using conventional biochemical testing and proteomic approaches (i.e. MALDI-TOF MS), and their correlation with increasing antibiotic resistance; however, the approach presented in this study would not be appropriate for detection of antibiotic resistance genes. Our study detected only the *erm* gene for MLS$_B$ resistance. In 18 CoNS isolates that were macrolide resistant, the *erm* gene was not detected. It is possible that these isolates harbour other resistance genes, such as macrolide efflux genes (*mef*), ATP-dependent efflux pump (*msrA/B*) or erythromycin esterase (*ereA/B*) [26].

We believe that rapid identification and drug susceptibility testing would better enable physicians to prescribe the appropriate antibiotic therapy, leading to better success in treatment and more prudent antibiotic usage, as well as be less conductive to staphylococcal resistance selection.

In conclusion, CoNS, which are capable of causing various types of infection, are highly prevalent in clinical specimens. They have ability to accumulate additional antibiotic resistance determinants. This study indicated that there are CoNS infections caused species other than *S. epidermidis* and *S. saprophyticus*, such as *S. haemolyticus*, *S. hominis* and *S. capitis*. These can be found in clinical specimens and may be considered to be causative agents of disease. Thus, identification and discrimination of *S. aureus*, CoNS and drug resistance are essential for effective antimicrobial chemotherapy, and they are also useful in surveillance, control and prevention of the spread of drug-resistant staphylococci.

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**Conflict of Interest**

None declared.

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**TABLE 4.** MLBS phenotype and genotype among 125 erythromycin-resistant CoNS isolates

| Gene   | Strains with MLBS phenotype, n (%) | Erlythromycin resistance, n (%) | Inducible MLBS | Constitutive MLBS | MSB |
|--------|-----------------------------------|--------------------------------|---------------|------------------|-----|
|        | MRCoNS | MSCoNS | MRCoNS | MSCoNS | MRCoNS | MSCoNS | MRCoNS | MSCoNS |
| *ermA* (8) | 7 (5.6) | 1 (0.8) | 0 | 0 | 7 (5.6) | 1 (0.8) | — | — |
| *ermC* (79) | 61 (48.8) | 18 (14.4) | 9 (7.2) | 2 (1.6) | 52 (41.6) | 16 (12.8) | — | — |
| *ermA* and *ermC* (20) | 16 (12.8) | 4 (3.2) | 5 (4) | 1 (0.8) | 12 (9.6) | 3 (2.4) | — | — |
| Without *ermA* and *ermC* (18) | 16 (12.8) | 2 (1.6) | 0 | 0 | 0 | 0 | 16 (12.8) | 2 (1.6) |
| Total | 100 (80) | 25 (20) | 14 (11.2) | 3 (2.4) | 70 (56) | 20 (16) | 16 (12.8) | 2 (1.6) |

CoNS, coagulase-negative staphylococci; MLBS, macrolide–lincosamide–streptogramin B; MSB, macrolide–streptogramin B; MRCoNS, methicillin-resistant CoNS (carries *mecA* gene); MRCoNS, methicillin-susceptible CoNS (no *mecA* gene).
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