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Antibiotic Resistance Patterns of Coagulase-Negative *Staphylococcus* (CoNS) Isolates from a Major Teaching Hospital in Kuala Lumpur, Malaysia

(Corak Kerintangan Antibiotik *Staphylococcus* Koagulase-Negatif (CoNS) Dipencilkan daripada Hospital Pengajar Utama di Kuala Lumpur, Malaysia)

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ABSTRACT

Coagulase-negative *Staphylococcus* (CoNS) species is a leading cause of nosocomial infection in patients with indwelling medical devices and immunocompromised patients. This study was conducted to determine the antibiotic resistance pattern of CoNS isolated from a major teaching hospital in Malaysia. A total of 43 CoNS isolates were collected from August to October 2018 at Hospital Canselor Tuanku Muhriz, Kuala Lumpur, Malaysia. Speciation of CoNS species was conducted by 16S rRNA sequencing. Antibiotic susceptibility test was performed using a standard procedure, and detection of staphylococcal cassette chromosome *mec* (SCC*mec*) elements and antibiotic resistance genes were conducted via multiplex polymerase chain reaction (PCR). Comparison of 16S rRNA sequences showed that 67.44% of the isolates were identified as *S. epidermidis*, followed by *S. haemolyticus* (11.63%), *S. hominis* (9.3%), *S. capitis* (4.65%), and other *Staphylococcus* sp. (6.98%). All the CoNS isolates were susceptible to linezolid and tedizolid, while most of them were resistant towards penicillin (86.05%), cefoxitin (69.77%), erythromycin (72.02%), and 88.37% of them were resistant to at least 3 antibiotics. The majority of CoNS harboured nontypeable SCC*mec* elements. *AacA-D* (95.5%) and *ermC* (78.6%) were the most commonly detected antibiotic resistance genes while no detection of *tetK*, *tetM* and *ermA* genes were observed. This study showed a high prevalence of multidrug-resistant CoNS in HCTM healthcare settings. Understanding CoNS resistance mechanism is warranted for intervention strategy.

Keywords: Coagulase-negative *Staphylococcus*; multi-drug resistance; SCC*mec* typing

ABSTRAK

Spesies *Staphylococcus* koagulase-negatif (CoNS) merupakan penyebab utama jangkitan nosokomium dalam kalangan pesakit yang menggunakan peralatan perubatan dan pesakit yang terimunokompromi. Penyelidikan ini telah dijalankan untuk menentukan corak kerintangan antibiotik dalam CoNS yang dipencilkan daripada hospital pengajar utama di Malaysia. Sejumlah 43 pencilan CoNS telah diambil daripada Hospital Canselor Tuanku Muhriz (HCTM), Kuala Lumpur, Malaysia daripada Ogos sehingga Oktober 2018. Penentuan spesies CoNS telah dijalankan menggunakan penjujukan 16S rRNA. Ujian kerentanan antibiotik telah dijalankan menggunakan prosedur piawai manakala pengesanan kromosom kaset *mec* stafilokokus (SCC*mec*) dan gen rintangan antibiotik dijalankan menggunakan tindak balas berantai polimerase multipleks(PCR). Perbandingan penjujukan 16S rRNA menunjukkan 67.44% pencilan CoNS adalah *S. epidermidis*, diikuti *S. haemolyticus* (11.63%), *S. hominis* (9.3%), *S. capitis* (4.65%) dan *Staphylococcus* sp. yang lain (6.98%). Semua CoNS rentan terhadap Linezolid and Tedizolid, manakala hampir semua rintang terhadap Penisilin (86.05%), Cefoksitin (69.77%), Eritromisin (72.02%) dan 88.37% adalah rintang terhadap sekurang-kurangnya 3 antibiotik. Majoriti CoNS mempunyai unsur SCC*mec* yang tidak dapat ditentukan. *AacA-D* (95.5%) dan *ermC* (78.6%) merupakan gen kerintangan yang paling kerap dijumpai manakala gen *tetK*, *tetM* dan *ermA* tidak ditemui. Kajian ini menunjukkan prevalens CoNS rintang terhadap pelbagai antibiotik adalah tinggi dalam persekitaran HCTM. Pemahaman mekanisme kerintangan CoNS adalah penting untuk strategi intervensi.

Kata kunci: Kerintangan pelbagai antibiotik; pengetipan SCC*mec*; *Staphylococcus* koagulase-negatif

Introduction

Coagulase-negative *Staphylococcus* sp. (CoNS) is a Gram-positive, non-motile species and human's skin normal flora of genus *Staphylococcus* that was once thought to be culture contaminant in the clinical laboratory setting (Becker et al. 2014). However, subsequent enhancement of CoNS isolation in the laboratory found the importance of CoNS in nosocomial infection particularly in patients with indwelling medical devices and immunocompromised patients (Hebeisen et al. 2019; Reers et al. 2016). Identification of CoNS can be performed by observation of colony morphology on selective media, biochemical test, nucleic acid comparison via sequencing of conserved 16S rRNA and recently via metabolomics approach (Argemi et al. 2015). CoNS infection usually occurs in patients who have inserted medical devices and immunocompromised patients that can lead to bacteraemia, endocarditis, osteomyelitis, sepsis, meningitis, and joint sepsis (Hitzenbichler et al. 2017). The ability of CoNS to form biofilm on fomites greatly enhances its pathogenicity to become an opportunistic pathogen in patients with the indwelling catheters (Seng et al. 2017). CoNS are different from *Staphylococcus aureus* as the former does not produce coagulase while the latter produce coagulase. The rise in antibiotic-resistant CoNS and methicillin-resistant CoNS (MRCoNS) that are mostly multi-drug resistant to multiple antimicrobial agents has further complicated treatment for nosocomial infection attributable to CoNS (Gu et al. 2013; Nascimento et al. 2015). The resistance mechanism of this pathogen to the antimicrobial agent is owed to the presence of *mecA* that encodes penicillin-binding protein 2a (PBP2a) on a mobile genetic element known as *Staphylococcus* cassette chromosome *mec* (SCC*mec*) (Liu et al. 2016). Expression of PBP2a leads to almost complete resistance to β-lactam antibiotics since this type of protein has a weaker affinity to bind to β-lactam antibiotics i.e. methicillin and penicillin (Leski et al. 2005). Besides *mecA* gene, another variant of *mecC* has been detected in CoNS (Loncaric et al. 2019). Regions of SCC*mec* elements include *mec* gene complex, *ccr* gene complex and 'junkyard' (J) region. The function of *ccr* gene complex is to integrate and excise SCC*mec* elements through the expression of recombinase (Liu et al. 2016). Unlike *ccr* gene complex in *S. aureus* that is usually detected as a single variant in one isolate, *ccr* gene complex of CoNS can be detected as multiple variants in one strain (Chen et al. 2017). Some strains also harbour SCC elements that carry genes for resistance to antiseptics i.e. mercury (*mer*), copper (*copB* and *copC*), arsenic (*ars*) and cadmium (*cad*) (Becker et al. 2014). Studies have also reported the large number of SCC*mec* elements in CoNS are non-typeable although *ccr* gene complex is detected, suggesting unique variants of SCC*mec* elements in CoNS have yet to be explored (Chen et al. 2017). To date, there are thirteen SCC*mec* types have been identified and described in *Staphylococcus* species (Baig et al. 2018). This mobile genetic element can be transferred horizontally among species that give rise to multi-drug resistant methicillin-resistant *S. aureus* (MRSA) (Otto et al. 2013). The previous study conducted in our hospital setting showed that *S. epidermidis* formed the largest CoNS found in clinical specimens, followed by non-typeable species and *S. saprophyticus* (Sani et al. 2011). SCC*mec* typing in *S. epidermidis* in our tertiary hospital showed large numbers of CoNS isolates harboured non-typeable SCC*mec* elements (Sani et al. 2014). Therefore, this study was aimed to determine antibiotic resistance patterns and antibiotic resistance genes of CoNS isolated from Hospital Canselor Tuanku Muhriz, Kuala Lumpur, Malaysia.

Materials and Methods

Culture of CoNS

Mannitol salt agar (MSA) was used to differentiate between CoNS and *Staphylococcus aureus*. Yellow colonies were observed for the culture of *S. aureus* while no colour change was observed for plates grown with CoNS colonies.

Antimicrobial susceptibility test

All CoNS isolates were tested for antimicrobial susceptibility by using the disk diffusion method on Muller Hinton agar (MHA) with adherence to Clinical Laboratory Standard Insitute (CLSI 2017) guidelines. Antimicrobial susceptibility test was conducted at Bacteriology Unit, Department of Diagnostic Laboratory Service, Hospital Canselor Tuanku Muhriz by using thirteen panels of antibiotics. They were penicillin, cefoxitin, erythromycin, clindamycin, gentamycin, trimethoprim-sulfamethoxazole, tetracycline, fusidic acid, rifampicin, mupirocin, ciprofloxacin, linezolid, and tedizolid.

Identification of CoNS species

Identification of CoNS species was conducted by sequencing 16S rRNA of bacteria. DNA was extracted by using the boiling method as previously described (Riyaz-Ul-Hassan et al. 2008). Amplification of

gene was conducted by using universal primers of 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3'). The final concentrations of primers and DNA were 0.4 µM and 50 ng/reaction, respectively. Primers and DNA were added to ExTen master mix (1st Base Asia, Singapore Science Park II, Singapore) for a final volume of 50 µL. PCR conditions were 94 °C (initial denaturation) for 2 min, followed by 35 cycles of 94 °C (1 min), 45 °C (1 min), and 72 °C (1 min), and final elongation at 72 °C for 10 min. All PCR products were run on 1.5% agarose gel to view amplified DNA bands and then sent for sequencing to 1st Base Asia (Singapore Science Park II, Singapore). Species identification was made by comparing 16S rRNA sequences with sequences from the BLAST database (National Centre for Biotechnology Institute 2021).

DETECTION OF ANTIBIOTIC RESISTANCE GENES IN CoNS VIA MULTIPLEX PCR

CoNS isolates that demonstrated resistance towards gentamicin, erythromycin, and tetracycline at the phenotypic level were further subjected to genotypic detection of antibiotic resistance genes. Detection of antibiotic resistance genes was performed as described previously (Safarpoor Dehkordi et al. 2017). Detection of *AacA-D*, *ermA*, *ermC,* and *tetK* was conducted via multiplex PCR while detection of *tetM* was conducted via singleplex PCR. Singleplex PCR for *AacA-D*, *ermA*, *ermC* and *tetK* was performed whenever DNA band was not detected from multiplex PCR reaction. All reactions were performed in a final volume of 25 µL that consisted of 50 ng of DNA, 12.5 µL of ExTEN master mix (1st Base, Singapore), 0.5 µM forward and reverse primers for each targeted gene, and distilled water.

SCC*mec* TYPING

Typing of SCC*mec* was conducted by multiplex PCR method as described previously (Ghaznavi-Rad et al. 2010). Briefly, eight primers were used to identify *ccr* gene complex. PCR reaction was performed in a final volume of 25 µL. PCR conditions were 95 °C for initial denaturation, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s and elongation at 72 °C for 90 s. Final elongation was performed at 72 °C for 10 min. PCR products were run on 1.5% agarose gel electrophoresis and viewed under UV light for DNA band detection. DNA samples without any bands or non-typeable in previous multiplex PCR were further subjected to a second multiplex PCR as described previously (Ito et al. 2014) for amplification of *ccr* gene and *mec* gene complex.

STATISTICAL ANALYSIS

Data analysis was conducted via Fisher's exact test. *P* value less than 0.05 was considered significant. All data were analysed by using Statistical Package for Social Science (SPSS) version 21.

RESULTS

PROFILING OF ANTIMICROBIAL SUSCEPTIBILITY IN CoNS ISOLATES

A total of 13 antibiotics were tested against CoNS isolates. Data analysis showed the prevalence of methicillin-resistant CoNS was significantly higher than methicillin-susceptible CoNS (P=0.0137; Fisher's exact test). All CoNS isolates were susceptible to linezolid and tedizolid (Table 1). CoNS had the highest resistance to penicillin 86.1% (37/43). This was followed by 74.4% (32/43) resistance to erythromycin, 69.8% (30/43) resistance to cefoxitin, 62.8% (27/43) resistance to fusidic acid, 51.2% (22/43) resistance to gentamycin, 48.8% (21/43) resistance to clindamycin, 44.2% (19/43) resistance to trimethoprim-sulfanethoxazole, 41.9% (18/43) resistance to rifampicin, 37.2% (16/43) resistance to ciprofloxacin, 34.9% (15/43) resistance to mupirocin, and 9.3% (4/43) resistance to tetracycline.

MULTIDRUG RESISTANCE IN CoNS

In this study, two isolates were resistant to only one antibiotic i.e., penicillin and 88.37% (38/43) of isolates were resistant to at least 3 antibiotics agents. Among these multidrug resistance isolates, one of them was resistant to 10 antibiotics, followed by four isolates were resistant to 9 antibiotics, and twelve isolates were resistant to 8 antibiotics. Among common drug-resistance combinations were penicillin-cefoxitin-erythromycin-clindamycin (46.5%, 20/43), followed by penicillin-cefoxitin-erythromycin-clindamycin-gentamycin (30.2%, 13/43) and penicillin-cefoxitin-erythromycin-clindamycin-gentamycin-trimethoprim/sulfamethoxazole (18.6%, 8/43).

TABLE 1. Profiling of antimicrobial susceptibility in 43 CoNS isolates from UKMMC

CoNS species (N=43)	Resistance to antibiotics (n)												
	PEN	FOX	ERY	CLIN	GEN	SXT	TET	FA	RIF	MUP	CIP	LINE	TED
S. epidermidis (n=29)	26	21	23	16	14	15	2	18	11	11	10	0	0
S. haemolyticus (n=5)	5	5	4	2	4	2	1	5	4	3	3	0	0
S. hominis (n=4)	3	3	2	2	2	2	1	2	2	0	1	0	0
S. capitis (n=2)	2	1	2	1	1	0	0	2	1	1	2	0	0
S. petrasii (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	0
S. lugdunensis (n=1)	0	0	0	0	0	0	0	0	0	0	0	0	0
S. warneri (n=1)	1	0	1	0	1	0	0	0	0	0	0	0	0

PEN=penicillin; FOX=cefoxitin; ERY=erythromycin; CLIN=clindamycin; GEN=gentamycin; SXT=trimethoprim-sulfamethoxazole; TET=tetracycline; FA=fusidic acid; RIF=rifampicin; MUP=mupirocin; CIP=ciprofloxacin; LINE=linezolid and TED=tedizolid

SPECIATION OF CoNS ISOLATES

Speciation of CoNS isolates were conducted by comparison of 16S rRNA sequences of bacteria. Comparison of 16S rRNA sequences with the sequences from BLAST database showed 67.44% (29/43) of CoNS isolates were *Staphylococcus epidermidis*, followed by 11.63% (5/43) of *Staphylococcus haemolyticus*, 9.3% (4/43) of *Staphylococcus hominis*, 4.65% (2/43) of *Staphylococcus capitis* and one isolate (2.3%) each for *Staphylococcus petrasii*, *Staphylococcus lugdunensis*, and *Staphylococcus warneri,* respectively.

DETECTION OF ANTIBIOTIC RESISTANCE GENES IN CoNS

Amplification of aminoglycoside resistance gene (*AacA-D*) showed 95.5% (21/22) of isolates harboured this gene. One isolate that lacked of *AacA-D* gene was *S. epidermidis*. On the other hand, amplification of erythromycin resistance genes (*ermA* and *ermC*) showed 78.6% (22/28) harboured *ermC*. Of which, 81.8% (18/22) of CoNS with detectable *ermC* were *S. epidermidis* while the rest was from *S. hominis* (2/22; 9.09%), *S. capitis* (1/22, 4.5%) and *S. haemolyticus* (1/22, 4.5%), respectively. No detection of *ermA* was observed among CoNS isolates that showed resistance to erythromycin at phenotypic level. Interestingly, no detection (0/4) of *tetK* or *tetM* was observed among CoNS isolates that showed resistance towards tetracycline at phenotypic level (Figure 1).

SCC*mec* TYPING AND CORRELATION WITH ANTIMICROBIAL SUSCEPTIBILITY OF CoNS

SCC*mec* typing was conducted based on multiplex PCR as described previously (Ghaznavi-Rad et al. 2010). From 43 CoNS isolates tested, 6 isolates were SCC*mec* type IV, followed by 3 isolates of SCC*mec* type III and one isolate each was type II and III, respectively. The

rest of isolates were untypeable (32/43). Among SCC*mec* type IV isolates, five isolates harboured SCC*mec* type IVb and another one harboured type IVc. Speciation of CoNS with typeable SCC*mec* showed that 72.8% (8/11) were *S. epidermidis* and the rest were two isolates of *S. haemolyticus* and one isolate of *S. hominis*, respectively (Table 2). Non-typeable SCC*mec* CoNS were further subjected to amplification of *ccr* and *mec* complex by

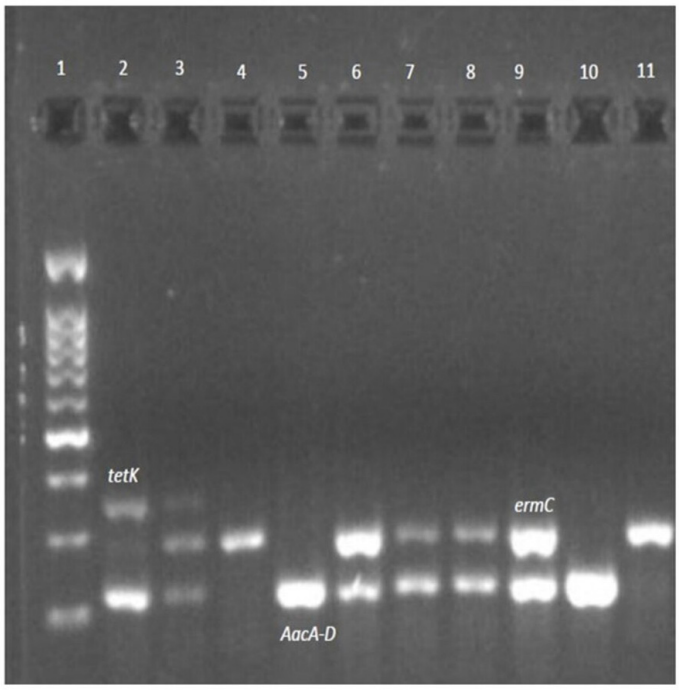

FIGURE 1. Antibiotic resistance genes amplified in CoNS isolates. Lane 1 shows products of 100 bp DNA ladder. Lane 2 to lane 11 were representative CoNS isolates. *tetK* was PCR product amplified at 360 bp, *AacA-D* was PCR product amplified at 227 bp and *ermC* was PCR product amplified at 299 bp

using method described previously (Ito et al. 2014). No detection of *mec* gene complex was observed in this group. However, amplification of *ccr* gene showed 34.4% (11/32) of isolates harboured *ccrAB3*, one isolate harboured both *ccrAB3* and *ccrAB2*, one isolate harboured both *ccrAB2* and *ccrAB1*, and the rest had no detectable *ccr* gene. Analysis of association between *ccr* gene and CoNS species showed all of CoNS that harboured single *ccrAB3* (11/32) were *S. epidermidis* while no single *ccrAB3* was detected in other staphylococci species (P=0.0002). Interestingly, one isolate that harboured both *ccrAB2* and *ccrAB1* was *S. hominis* while one isolate that harboured both *ccrAB3* and *ccrAB2* was *S. epidermidis*.

Analysis of SCC*mec* type and antibiotics susceptibility profiling showed that 54.5% (6/11) of CoNS that harboured SCC*mec* type IV were resistant to at least five antibiotics. A total of three isolates that harboured SCC*mec* type V were resistant to at least 7 antibiotic agents. Interestingly, one isolate with SCC*mec* type II/III was resistant to 10 antibiotic agents.

DISCUSSION

Antimicrobial susceptibility tests on CoNS isolates showed that 88.37% of them were multidrug-resistant isolates (resistance to three or more antimicrobial agents). This finding was also consistent with another study where the majority of CoNS isolates were found to be multidrug-resistant towards antibiotics (Maleki et al. 2019). High frequency of CoNS isolates were resistant to β-lactam, macrolide, fucidane, and aminoglycosides type of antibiotics, indicating the less efficacy of this type of antibiotics in treating infections caused by CoNS from our setting. Linezolid and tedizolid remain as

antibiotics of choice for treatment of CoNS as no isolates demonstrated resistance to these two antibiotics. Similar to other studies conducted previously, blood culture remains as the major source for CoNS (Castanheira et al. 2010; Weinstein et al. 1997). Identification of CoNS species by using 16S rRNA showed that *S. epidermidis* remains as a major species of CoNS isolated in our setting. Studies conducted previously in Malaysia also found *S. epidermidis* to be the most frequently isolated from clinical settings (Sani et al. 2011).

TABLE 2. Association between SCC*mec* types and antimicrobial susceptibility profiles of CoNS

CoNS species	SCC*mec* type	Antimicrobial susceptibility profile
S. epidermidis	IVc	PEN, FOX, ERY, CLIN, SXT, FA
S. epidermidis	IVb	PEN, FOX, ERY, CLIN, GEN, SXT, RIF
S. epidermidis	IVb	PEN, FOX, ERY, CLIN, GEN, SXT, FA, RIF
S. haemolyticus	IVb	PEN, FOX, GEN, SXT, FA, RIF, CIP
S. epidermidis	IVb	PEN, FOX, ERY, CLIN, SXT, FA, RIF, MUP
S. epidermidis	IVb	PEN, FOX, ERY, GEN, SXT, FA, RIF, CLIN
S. epidermidis	V	PEN, FOX, ERY, CLIN, GEN, SXT, FA, RIF, MUP
S. epidermidis	II/III	PEN, FOX, SXT, TET, FA, RIF, MUP
S. epidermidis	V	PEN, FOX, ERY, CLIN, GEN, SXT, FA, CIP
S. haemolyticus	V	PEN, FOX, ERY, CLIN, GEN, TET, MUP, CIP
S. hominis	II/III	PEN, FOX, ERY, CLIN, GEN, SXT, TET, FA, RIF, CIP

PEN=penicillin; FOX=cefoxitin; ERY=erythromycin; CLIN=clindamycin; GEN=gentamycin; SXT=trimethoprim-sulfamethoxazole; TET=tetracycline; FA=fusidic acid; RIF=rifampycin MUP=mupirocin; CIP=ciprofloxacin

Antibiotic resistance genes of tetracycline, erythromycin, and aminoglycoside were chosen to be detected because they are essential antibiotics used for the treatment of CoNS infection in our healthcare setting (Ministry of Health Malaysia 2014). Detection of antibiotic resistance genes of CoNS showed that almost all of them harboured aminoglycoside resistance gene (*AacA-D*) and a high prevalence of *ermC* was observed. This is consistent with a recent study in which a high prevalence of *AacA-D* was observed in CoNS isolates (Chabi & Momtaz 2019) although they observed a low prevalence of *ermC*. Resistance mediated by *tetK* involves protein efflux mechanism while resistance mediated by *tetM* involves ribosomal protection (Foster 2017). Interestingly, no detection of tetracycline genes (*tetK* or *tetM*) was observed, suggesting a different mechanisms of tetracycline resistance or different variants of *tet* genes in our healthcare setting.

In this study, we were also interested to observe the type of SCC*mec* element harboured among CoNS isolates in our hospital setting. A previous study conducted on *S. epidermidis* isolates in our centre showed that almost 40% of them harboured non-typeable SCC*mec* elements (Sani et al. 2014). Typing of SCC*mec* elements by using multiplex PCR as conducted previously (Ghaznavi-Rad et al. 2010) showed that SCC*mec* elements were only successfully typed in 11 isolates. SCC*mec* elements were not successfully typed in the majority of CoNS isolates, suggesting a unique gene arrangement of SCC*mec* in CoNS, compared to *S. aureus*. Previous SCC*mec* typing

(Ghaznavi-Rad et al. 2010) was performed in *S. aureus*, instead of CoNS isolates. The majority of CoNS isolates harboured SCC*mec* type IV. Interestingly, recent studies conducted in our hospital setting showed the majority of MRSA isolates harboured SCC*mec* type IV (Ismail et al. 2021; Sukri et al. 2021). In contrast, majority of the MRSA isolates from our hospital setting a decade ago harboured SCC*mec* type III (Noordin et al. 2016). This result indicates the clonal replacement of MRSA may taking place in our hospital setting and it is important because CoNS serves as a reservoir for horizontal transfer of SCC*mec* elements to MRSA, suggesting future study on the mechanism and to what extent the transfer of SCC*mec* from different species of *Staphylococcus* gives rise to multidrug resistance bacteria. As SCC*mec* type IV is usually associated with *Staphylococcus* species from the community, this finding indicated the presence of CoNS isolates from the community in our hospital setting. Furthermore, CoNS that harboured SCC*mec* type IV elements also demonstrated resistance to multiple antibiotics. The mechanism on why CoNS with SCC*mec* type IV demonstrated resistance to multiple antibiotics could not be ascertained from this study. However, MRSA harbouring SCC*mec* type IV has been shown to be able to survive desiccation and harsh environment better than the MRSA harbouring SCC*mec* type III (Knight et al. 2012). Although the study was conducted on MRSA, this factor may partially explain the evolutionary 'fitness' of CoNS harbouring SCC*mec* type IV to become resistant to multiple antibiotics. Nevertheless, future study that employs genomics profiling should be conducted to unravel the molecular mechanism of multiple antibiotics resistance in CoNS.

Conclusion

In conclusion, CoNS species remains a serious health threat in our hospital setting as they demonstrated resistance to multiple antibiotics. Typing of SCC*mec* elements in CoNS showed that type IV was prominent in our setting, suggesting the presence of CoNS isolates from the community. Unsuccessful typing of SCC*mec* elements in the majority of CoNS isolates from our hospital setting suggests a unique arrangement of SCC*mec* elements in CoNS and future typing by using other methods are warranted.

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