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High prevalence of methicillin-resistant coagulase-negative staphylococci isolated from a university environment in Thailand

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Summary. The present study was conducted to isolate and characterize the molecular epidemiology of the methicillin-resistant staphylococci in the general university environment, where all five locations; the library, restrooms, canteens, computer rooms and outdoor surfaces, are in common use by a large population of students. We used Mannitol Salt Agar (MSA) supplemented with 4 µg/ml of oxacillin to screen the methicillin-resistant staphylococci. The species level was identified by PCR of *rdr* (*Staphylococcus epidermidis*), *groESL* (*Staphylococcus haemolyticus*) and *nuc* (*Staphylococcus aureus* and *Staphylococcus warneri*) genes and DNA sequencing of *tuf* and *dnaJ* genes. The susceptibility patterns of the isolates were determined using the disk diffusion method. Antibiotic and disinfectant resistance genes, together with SCC*mec* types, were detected by the PCR method. The methicillin resistant-staphylococci were isolated from 41 of 200 samples (20.5%), and all of them were found to be methicillin-resistant coagulase negative staphylococci (MR-CoNS). The library had the highest percentage of contamination, with 43.3% of the samples found to be contaminated. All isolates belonged to 6 different species including *S. haemolyticus*, *S. epidermidis*, *S. warneri*, *S. cohnii*, *S. saprophyticus* and *S. hominis*. The antimicrobial resistance rates were highest against penicillin (100%), then cefoxitin (73.1%), erythromycin (73.1%) and oxacillin (68.3%). Altogether, the isolates were approximately 61.0% multidrug resistant (MDR), with the *S. epidermidis* isolates being the most multidrug resistant ($P < 0.05$). The prevalence of the *qacA/B* gene was detected in 63.4% of the isolates, and SCC*mec* could be typed in 43.9% (18/41) of the isolates. The type range was: II (n = 1), IVd (n = 1), I (n = 2), V (n = 6), IVa (n = 8) and untypeable (n = 23). This result indicates that these university environments are contaminated with methicillin-resistant coagulase negative staphylococci that carry various SCC*mec* types and high rate of disinfectant resistance genes. [**Int Microbiol** 20(2):65-73 (2017)]

Keywords: *Staphylococcus* spp. · methicillin-resistant coagulase negative staphylococci · drug resistance · gene *qacA/B* · Phitsanulok (Thailand)

Introduction

Staphylococci, particularly *Staphylococcus aureus*, *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. hominis*, and *S. lugdunensis* are medically important pathogens which cause nosocomial and community infections [29]. Staphylococci are classified into coagulase-positive staphylococci known as *S. aureus* and coagulase-negative staphylococci (CoNS) such as *S. epidermidis*, *S. saprophyticus*, *S. haemolyticus*, *S. hominis* and another 49 species [28]. Most strains of these bacteria have developed methicillin resistance and

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are therefore called methicillin-resistant *S. aureus* (MRSA) and methicillin resistant coagulase negative staphylococci (MR-CoNS), which constitute a major health problem. More recently, the number of reports of community-acquired MRSA (CA-MRSA) has been rapidly increasing. However, infections caused by MR-CoNS in the community have not been reported, but the gene transfer from these bacteria to MRSA has been identified [23].

The resistance in MRSA and MR-CoNS is caused by the acquisition of the *mecA* gene that encodes a modified penicillin-binding protein 2a (PBP2a) which has a low binding affinity for all beta-lactam antibiotics. The *mecA* gene is located within the *mec* operon carried by staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* are classified into 11 different types and various subtypes [46]. SCC*mec* type I, II and III are carried by hospital-acquired MRSA (HA-MRSA) and SCC*mec* type IV, V and VI are carried by community-acquired MRSA (CA-MRSA) [6]. In contrast to MRSA, the distribution of SCC*mec* types in MR-CoNS is varied, depending on the human host and the geographical locations from where the isolates were obtained [40]. Moreover, the variety of other antibiotic and disinfectant resistance genes such as erythromycin resistance genes (*erm*) and the quaternary ammonium compound resistance gene (*qacA/B*) were either identified in plasmids of, or by mobile genetic elements of, staphylococci [44].

Recent studies have reported that a wide variety of different high-touch environmental surfaces in public facilities, universities, microbiological and computer laboratories, day-care centers, prisons and clinics are a potential reservoir of MRSA and drug resistance genes [7]. However, environmental colonization to the spread of MR-CoNS is poorly reported, although Widerström and coworkers [38] found that the hospital-acquire methicillin-resistant *S. epidermidis* was spread from environmental fomites to patients in Intensive Care Units. Xu, Mkrtchyan and Cutler [40] reported that 21% of hotel samples were contaminated with MR-CoNS, and Mkrtchyan and coworkers [20] found that staphylococci are the most predominant bacteria in non-hospital restrooms, in the UK. Moreover, 23 MR-CoNS were isolated from 7 beach sites in Washington State by Soge and coworkers [33]. We have been able to find only these three papers published on the matter, therefore there is little information available on the prevalence and molecular epidemiology of methicillin-resistant staphylococci isolated from non-hospital environments, and this is especially so in the case of Thailand.

Therefore, exploration of this information has become an important issue demanding investigation, which was the purpose of the current study. We isolated and identified methicillin-resistant staphylococci at the specie level, from a university environment in Thailand, and determined the antimicrobial susceptibility pattern, detected the antibiotic and disinfectant resistance genes, and characterized the SCC*mec* types of all isolates.

Materials and methods

Population and samples. The sample size of the study was calculated using the Sample Size Determination in Health Studies Software of the World Health Organization [17]. A total of 200 swab samples were randomly collected from 5 locations of a university community in Thailand, including computer rooms (computer mouse, computer earpieces, computer keyboards and computer power buttons) (n = 40); restrooms (door handles, washbasins, washbasin areas, urinary taps and toilets) (n = 50), the library (books, escalators and tables) (n = 30), canteens (tables, bank notes and coins used for payment, ATM machines and water dispensers) (n = 40), and outdoor surfaces (handrails, exercise machines, public buses) (n = 40). The sample collection was carried out from September to December 2015 with temperature ranging from 31 ºC to 32 ºC at the study area.

Bacterial isolation of methicillin-resistant staphylococci. The samples were collected using cotton swabs soaked in 0.85% normal saline, and then placed in transfer media (2% of skim milk powder, 3% of tryptone soya broth (TSB), 0.5% glucose and 10% glycerol). The swab samples were enriched in TSB with shaking at 180 rpm at 37 ºC for 18–24 h. Then, one loopful of overnight culture was streaked on MSA with 4 µg/ml of oxacillin and incubated at 30 ºC for 48–72 h for a primary screening for methicillin-resistant staphylococci base on the method of Lally et al. [15]. All colonies were selected for further identification using Gram's stain, catalase and coagulase tests. All isolates were subsequently confirmed as staphylococci by the PCR method amplified by 16S rRNA specific primers [13]. Methicillin-resistance was further confirmed by oxacillin disk (1 µg), cefoxitindisk (30 µg) and the PCR method to detect *mecA* gene. *Staphylococcus aureus* COL was used as positive control of this method.

Identification of methicillin-resistant staphylococci species. *Staphylococcus aureus*, *S. epidermidis*, *S. haemolyticus* and *S. warneri* were distinguished from other species by the PCR method based on the specific primer of *nuc* (*S. aureus* and *S. warneri*), *rdr* (*S. epidermidis*) and *groESL* (*S. haemolyticus*) as described by Schmidt et al. [28]. A specific gene of each species was sequenced to ensure the absence of bias in our method. The remaining isolates that could not be identified by PCR were identified by *tuf* and *dnaJ* gene sequencing using Sanger Sequencing Method, according to the methods described by Loonen et al. [16] and Shah et al. [30]. The PCR products were purified using an RBC purification kit and sequenced using Applied Biosystems. Sequence similarities of *tuf* and *dnaJ* genes > 97% were used to identify isolates at the species level. The primer sets of *nuc*, *rdr*, *groESL*, *tuf* and *dnaJ* genes are shown in Table 1.

Table 1. Primers for antibiotic resistance gene amplification and bacterial identification

Primers	Oligonucleotide sequence (5'-3')	Tm (°C)	Product size (bp)	Reference
nuc-F(*S. aureus*)	TCGCTTGCTATGATTCTGG	55.2	359	[27]
nuc-R(*S. aureus*)	GCCAATGTTCTACCATAGC	55.2		
nuc-F(*S. horminis*)	TACAGGGCCATTTAAAGACG	56.4	177	[10]
nuc-R(*S. hominis*)	GTTTCTGGTGTATCAACACC	56.4		
nuc-F*(S. warneri)*	CGTTTGTAGCAAAACAGGGC	58.4	999	[10]
nuc-R*(S. warneri)*	GCAACGAGTAACCTTGCCAC	60.5		
rdr-F	AAGAGCGTGGAGAAAAGTATCAAG	61.8	130	[32]
rdr-R	TCGATACCATCAAAAAGTTGG	61.8		
groESL-F	GGTCGCTTAGTCGGAACAAT	57.8	271	[4]
groESL-R	CACGAGCAATCTCATCACCT	57.8		
tuf-F	CCAATGCCACAAACTCGTGA	58.4	480	[16]
tuf-R	CAGCTTCAGCGTAGTCTAATAATTTACG	65.7		
dnaJF	GCCAAAAGAGACTATTATGA	52.3	920	[30]
dnaJR	ATTGYTTACCYGTTTGTGTACC	56.6		
mecA-F	TGGCTATCGTGTCACAATCG	58	310	[26]
mecA-R	GTTCTCTCATAGTATGACGTCC	58		
ermA-F	AAGCGGTAAACCCCTCTGA	56.7	190	[34]
ermA-R	TTCGCAAATCCCTTCTCAAC	55.2		
ermB-F	AATCGTCAATTCCTGCATGT	55.9	142	[3]
ermB-R	TAATCGTGGAATACGGGTTTG	55.9		
ermC-F	AATCGTCAATTCCTGCATGT	53.2	299	[34]
ermC-R	TAATCGTGGAATACGGGTTTG	55.9		
qacA/B-F	GCAGAAAGTGCAGAGTTCG	57.3	361	[44]
qacA/B-R	CCAGTCCAATCATGCCTG	56.1		

Determination of antimicrobial susceptibility patterns. Antimicrobial susceptibility of all isolated methicillin-resistant staphylococci was tested using a standard disk diffusion test against fifteen antibiotics: penicillin (P, 10 units), clindamycin (DA, 2 µg), chloramphenicol (C, 30 µg), gentamicin (CN, 10 µg), erythromycin (E, 15 µg), cefoxitin (FOX, 30 µg), sulfamethoxazole/trimethoprim (SXT, 1.25/23.75 µg), oxacillin (OX, 1 µg), vancomycin (VA, 30 µg), rifampicin (RD, 5 µg), linezolid (LZD, 30 µg), mupirocin (MUP, 5 µg), ciprofloxacin (CIP, 5 µg), fusidic acid (FD, 10 µg) and novobiocin (NV, 5 µg). The plates were incubated at 35 ºC for 24 h. *Staphylococcus aureus* NCTC10442 was used as positive control and the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute 2014. All isolates were categorized as MDR when they were resistant to at least three classes of antibiotics [19].

Detection of antibiotic and disinfectant resistance genes. The methicillin-resistance gene (*mecA*) was detected according to the method described by Kitti, Boonyonying and Sitthisak [12]. *Staphylococcus aureus* COL was used as positive control of this detection. Other antibiotic resistant genes, including *erm*(A), *erm*(B), *erm*(C) *and qacA/B,*were detected by PCR modified from the method described in [1,41,44]. The primers used are shown in Table 1. The absence of bias was ensured by the sequencing of each gene in the representative isolates. All PCR products were visualized using gel electrophoresis with 1% agarose gel stained with 0.5% ethidium bromide.

Characterization of SCC*mec* types. SCC*mec* types of all isolates were characterized according to the method of Zhang et al. [43]. *Staphylococcus aureus* NCTC10442, *S. aureus* JCSC10442 , *S. aureus* WIS and *S. aureus* isolated from our previous study [36] were used as reference strains of SCC*mec* Type I, II, IVa, IVb and V. The amplicons were visualized using gel electrophoresis with 1% agarose gel stained with 0.5% ethidium bromide.

Statistical analysis. All data was analyzed using Stata 12.0 (Stata Corporation, USA). The analysis of frequency (Chi-square test; $P < 0.05$) was used as the statistic to compare the MDR and antimicrobial susceptibility patterns among MR-CoNS species. The association between MR-CoNS prevalence and environmental locations was calculated using logistic regression ($P < 0.05$).

Results

The prevalence of methicillin-resistant staphylococci. We used MSA, supplemented with 4 µg/ml of oxa-

Table 2. The association of MR-CoNS prevalence with environmental locations

Locations[a]	N	Positive MR-CoNS (%)	Negative MR-CoNS (%)	OR	95% CI	*P*-value
LB	30	13 (43.3)	17 (56.7)			
RR	50	14 (28.0)	36 (72.0)	0.50	0.19, 1.31	
CN	40	8 (20.0)	32 (80.0)	0.32	0.11, 0.94	
CR	40	5 (12.5)	35 (87.5)	0.18	0.05, 0.60	
US	40	1 (2.5)	39 (97.5)	0.03	0.00, 0.27	<0.001*

[a]LB: Library, RR: Restroom, CN: Canteen, CR: Computer room and US: Outdoor surfaces

OR: odds ratio. CI: confidence interval. *Significant at $P < 0.001$

cillin, to screen for methicillin-resistant staphylococci and confirmed by oxacillin disk (1 µg), cefoxitin disk (30 µg) and *mecA* gene detection. Among the 200 samples, 41 (20.5%) were identified as methicillin-resistant staphylococci, while all of them were MR-CoNS. The library was the most contaminated, with 43.3% of the locations tested showing staphylococci contamination. The next most contaminated were the restrooms (28%), canteens (20%), computer rooms (12.5%) and outdoor surfaces (2.5%).

Logistic regression was performed to analyze the association between the environmental locations and MR-CoNS prevalence. Overall, the environmental locations tested in our study were significantly associated with colonization of MR-CoNS ($P < 0.001$). More precisely, the library was the most contaminated region (Table 2).

Species distribution. The specie level of all isolates was identified by our combined method of biochemical test, PCR and DNA sequencing. All 41 isolates of MR-CoNS belonged to 6 different species including *S. haemolyticus* (41.5%), *S. epidermidis* (36.6%), *S. warneri* (12.2%), *S. cohnii* (4.9%), *S. saprophyticus* (2.4%) and *S. hominis* (2.4%) (Fig. 1).

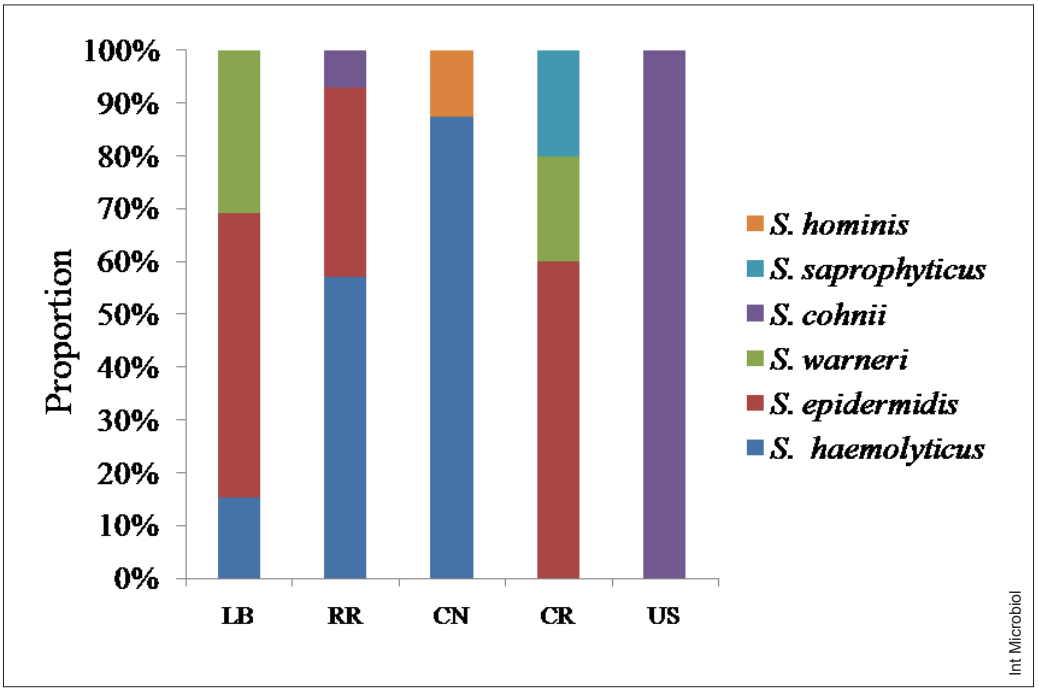

Fig. 1. Prevalence of MR-CoNS and species distribution by environmental locations (LB: Library, RR: Restroom, CN: Canteen, CR: Computer room, and US: Outdoor surfaces.)

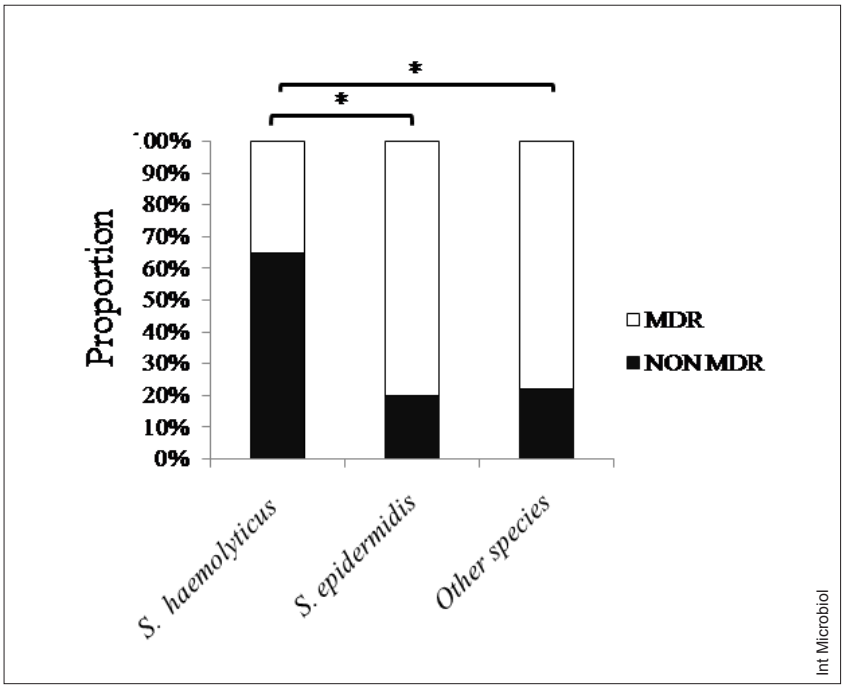

Fig. 2. Differences among MDR and NON MDR pattern of each species. *Significant at $P < 0.05$.

Antimicrobial susceptibility pattern. All isolates were resistant to penicillin (100%), cefoxitin (73.1%), erythromycin (73.1%), oxacillin (68.3%), sulfamethoxazole/trimethoprim (29.3%), fusidic acid (22.0%), clindamycin (14.6%), ciprofloxacin (12.2%), chloramphenicol (9.8%), novobiocin (9.8%), gentamicin (4.9%), rifampicin (2.4%), mupirocin (2.4%). All isolates were susceptible to vancomycin and linezolid. We found most of the isolates (approximately 61.0%) were MDR. We divided all 41 isolates into three species groups, *S. haemolyticus*, *S. epidermidis* and other species. Then, we compared the resistance rate of each antibiotic among these species using chi-square test. Significantly, the prevalence of MDR in each group was different ($P < 0.05$). *Staphylococcus epidermidis* and other species (*S. warneri*, *S. cohnii*, *S. hominis* and *S. saprophyticus*) were more associated with MDR than was *S. haemolyticus* ($P < 0.05$) (Fig. 2). *Staphylococcus haemolyticus* was more resistant to clindamycin than were *S. epidermidis* and other species ($P < 0.05$) (Table 3).

Antibiotic and disinfectant resistance genes. Gene *mecA* was detected in all MR-CoNS isolates by the PCR method. Due to the high resistance to erythromycin, its resistance genes: *erm*(A), *erm*(B) and *erm*(C) were also detected. Only *erm*(C) was detected in 14.6% of isolates and 20.0% of erythromycin resistant isolates, indicating the low correlation between the phenotypic pattern and the causative genes. We also detected *qacA/B* gene, the gene encoding resistance to several antiseptics and disinfectants. A high proportion (63.4%) of all isolates was positive with this gene.

SCC*mec* types. All isolates were subjected to characterization by their SCC*mec* types using multiplex PCR. Among all the isolates, 43.9% (18/41) were characterized as SCC*mec* type I (n = 2), II (n = 1), IVa (n= 8), IVd (n = 1) and V (n = 6). We found 56.1% (23/41) were untypeable SCC*mec* types. The distribution of SCC*mec* types in each species is shown in Table 4.

Discussion

To strengthen the understanding about the dissemination of methicillin-resistant staphylococci within non-health care environments, we examined isolates from a university environment in Thailand, and identified the specie levels in those isolates. Most of the MR-CoNS (43.3%) isolates in the study were obtained from items in the library, such as books and study tables. Hence, MR-CoNS can be easily spread by the simple act of reading a book at a table in the library.

Table 3. The comparison of *qacA/B* gene and antimicrobial resistance patterns in each MR-CoNS species

Antibiotics	*S. haemolyticus* n = 17 (%)	*S. epidermidis* n = 15 (%)	Other species n = 9 (%)	Total n = 41 (%)	*P*-value
qacA/B	12 (70.6)	9 (60.0)	5 (55.6)	26 (63.4)	0.767
Oxacillin	12 (70.6)	12 (80.0)	4 (44.4)	28 (68.3)	0.187
Cefoxitin	12 (70.6)	13 (86.7)	5 (55.6)	30 (73.1)	0.238
Erythromycin	13 (76.5)	10 (66.7)	7 (77.8)	30 (73.1)	0.773
Sulfamethoxazole/ Trimethoprim	4 (23.5)	3 (20.0)	2 (22.2)	9 (22.0)	0.971
Chloramphenicol	0 (0.0)	2 (13.3)	2 (22.2)	4 (9.6)	0.162
Rifampicin	0(0.0)	1 (6.7)	0 (0.0)	1 (2.4)	0.411
Gentamicin	1 (5.9)	1 (6.7)	0 (0.0)	2 (4.9)	0.740
Fusidic acid	5 (29.4)	1 (6.7)	0(0.0)	6 (14.6)	0.071
Clindamycin	10 (58.8)	1 (6.7)	1 (11.1)	12 (29.3)	<0.05*
Mupirocin	0 (0.0)	0 (0.0)	1 (11.1)	1 1 (2.4)	0.162
Novobiocin	1 (5.9)	3 (20.0)	0 (0.0)	4 (9.6)	0.218
Ciprofloxacin	1(5.9)	2 (13.3)	2 (22.2)	5 (12.2)	0.473
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA
Linezolid	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	NA

*Significant at $P < 0.05$.
NA = not analyzed.

Using our screening method, none of the isolates was identified as MRSA. Like in a previous report, on a study conducted in India, MRSA was not recovered from a hospital environment [31]. To ascertain the specie distribution of MR-CoNS isolates in this study, we identified that 6 different species were presented in all of the isolates (Fig. 1). These species are similar to the CoNS obtained from non-hospital environments [20,40], clinical specimens [14,25], healthy adult volunteers [2] and chicken meat [22]. However, the prevalence of each species was different among these specimens. *Staphylococcus epidermidis* was categorized as the predominant species of CoNS isolated from clinical and commensal samples, while *S. haemolyticus* was found to have the highest prevalence in non-healthcare environments.

Table 4. Distribution of SCC*mec* types among MR-CoNS isolated from the university environment. The values in the table indicate the number of each SCC*mec* type in each species

MR-CoNS Species	SCC*mec*					
	I	II	IVa	IVd	V	Untypeable
S. haemolyticus	0	1	1	0	3	12
S. epidermidis	0	0	4	1	1	9
S. warneri	0	0	3	0	2	0
S. cohnii	2	0	0	0	0	0
S. saprophyticus	0	0	0	0	0	1
S. hominis	0	0	0	0	0	1
Total	2	1	8	1	6	23

In contrast, the species distribution on our study was different from CoNS contaminating fermented food and starter cultures [42].

More than 60% of all isolates were resistant to penicillin, cefoxitin, and erythromycin. Two notable antimicrobial resistance patterns of these isolates were found. First, 61.0% of all isolates was identified as MDR, which higher than the rate of staphylococci MDR isolated from commensal specimens [21], and about 10 times more than the prevalence reported by Cavanagh et al. [2]. Second, *S. epidermidis* and other species (*S. warneri*, *S. cohnii*, *S. saprophyticus* and *S. hominis*) were all significantly associated with MDR, and all higher than *S. haemolyticus*. To our knowledge, this is the first comparison of MDR patterns among MR-CoNS collected from non-healthcare environments. However, studies with larger samples are needed to confirm these findings.

We found 11 *mecA* positive isolates that were not resistant to oxacillin and cefoxitin. This may be explained by: (a) not all *mecA* positive staphylococci are resistant to oxacillin due to the low expression of PBP2a causing the low levels of minimum inhibitory concentration (MIC) [40], and (b) MR-CoNS can be incorrectly characterized by cefoxitin disk diffusion (35)**.** Additionally, 63.4% of all isolates in the present study carried the *qacA/B* gene. This prevalence was higher than the rate of *qacA/B* gene carried by CoNS isolated from surgical sites [37], nurses and the general population in Hong Kong [44].

According to previous studies, the SCC*mec* types in MR-CoNS are more diverse than in MRSA [11]. Hanssen et al. [8] revealed that staphylococcal strains from the same geographical region carry identical *ccr* genes and differ from sequences of strains from other regions. This agreement supports the evidence of horizontal SCC*mec* gene transfer among staphylococcal strains [9,46]. All 41 MR-CoNS isolates in our study were characterized for their SCC*mec* types, and we found that most of them (23/41) were assigned as untypeable. This result correlated with *S. hominis* isolates from blood that carried the high rate (82%) of untypeable SCC*mec* types [18]. These untypeable SCC*mec* were suspected to carry novel SCC*mec* types as described previously [24,45]. However, the bias may have occurred due to the use of primer sets developed for *S. aureus*. In addition to this result, 4/8 of SCC*mec* IVa and 1/1 of SCC*mec* IVd belonged to methicillin-resistant *S. epidermidis* (MRSE). This supported the description of Wisplinghoff et al. [39] and Du et al. [5] that found SCC*mec* IV in most MRSE strains.

In conclusion, the university environments such as library, canteen, restrooms and computer room are the essential reservoirs of MR-CoNS. The species distribution of these isolates was similar to the strains isolated from clinical and commensal specimens. We demonstrated the high prevalence of quaternary ammonium resistance gene of these MR-CoNS and most of the isolates were multidrug resistant bacteria. This finding provided useful information to support disease prevention strategies against staphylococcal infections. People should be careful when touching these surfaces because they can be the carrier of these high-antibiotic resistance bacteria to other people. Hand washing activity should be usually practiced to eliminate this reservoir.

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Competing interests. None declared.

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